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Prepared by **Mark J. Sercovich**

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INTRODUCTION

Human immunodeficiency virus type I (HIV-1) infection causes acquired immunodeficiency syndrome (AIDS), the most globally devastating viral disease of the past 25 years. Development of effective HIV-1 preventative and therapeutic regimens has proven exceedingly difficult, as the virus has evolved sophisticated mechanisms for thwarting control efforts. A detailed understanding of HIV-1 molecular biology is therefore necessary in order to generate the effective and inexpensive prevention and treatment strategies required for AIDS pandemic curtailment.

HIV-1 optimizes its transmissibility and propagation through continual change and coordination of its components' functions and life cycle processes with one another and with those of cellular components and processes. Comprehending the molecular bases for HIV-1's abilities to manipulate host cell components and processes is key to the identification of the virus's vulnerabilities. This thesis focuses on one identified effect, G_2/M cell cycle arrest induction (1-5), of one highly conserved HIV-1 component, viral protein R (Vpr) (6, 7). A mechanistic understanding of this function is important because arrest at this cell cycle stage provides a selective advantage for the virus: transcription from the viral promoter more active during G_2, allowing for increased viral replication (8-14). Other reasons for the selective advantage of G_2/M arrest, *e.g.* prevention or delay of cell death by "mitotic catastrophe" or apoptosis, are also possible (3, 15-23). Covering scientific publications through November 2005, this thesis explores the state of knowledge of the mechanism(s) underlying Vpr's ability to induce G_2/M cell cycle arrest. The author's goal is to provide a disinterested analysis of the available mechanistic

models and their supporting data with the hope of being helpful to the reader in some manner.

BACKGROUND

To provide a context for ensuing sections, this section provides cursory overviews of the HIV-1 life cycle and of Vpr structure and proposed functions other than cell cycle arrest induction. Excellent, detailed reviews covering these topics are available elsewhere [examples include (24, 25)].

HIV-1 Life Cycle Overview

HIV-1 is a member of the *Lentivirus* genus of the *Orthoretrovirinae* subfamily of *Retroviridae*. The assignment of the "retrovirus" family name was based on the major distinguishing feature of its members, their ability to generate double-stranded DNA copies from RNA genomic templates by virtue of a provirus-encoded, virion-associated reverse transcriptase (RT). Each retrovirus additionally encodes an integrase (IN) to catalyze essential steps in the DNA recombination process for insertion of viral DNA into the host cell genome. Based on genome structure, retroviruses are classified as either simple or complex. All retroviruses possess 5'- and 3'-long terminal repeats (LTRs); between the LTRs, however, simple retroviruses possess only three genes, *gag*, *pol*, and *env*, while complex ones possess additional, auxiliary genes. HIV-1 is a complex retrovirus with six auxiliary genes: *tat*, *rev*, *vif*, *vpu*, *nef*, and *vpr*.

The HIV-1 life cycle begins with viral attachment to the host cell via direct interaction between the viral envelope glycoprotein complex [gp120-gp41 or surface

(SU)-transmembrane (TM)] and specific host cell surface receptors (CD4) and co-receptors (CXCR4 for macrophage-tropic strains; CCR5 for those infecting lymphocytes). Viral protein-mediated fusion of the viral and cellular envelopes ensues, allowing access of the viral core complex to the cytoplasm of the infected cell. Core-associated RT then catalyzes the production of a linear, double-stranded DNA copy of the RNA genome, and this DNA is translocated into the nucleus as part of the viral pre-integration complex (PIC). The PIC, containing IN and other core-associated viral proteins, contacts chromatin, permitting integration of the viral DNA into the host genome via IN-catalyzed recombination. The integration process appears to have few, if any, site specificity requirements, and the role(s) of host DNA damage recognition and repair components in the integration process is/are controversial.

Post-integration, retroviral DNA is termed provirus and its full-length transcription is catalyzed by cellular RNA polymerase II (pol II). Alternative splicing of the primary RNA transcript at single or multiple sites produces more than 30 different mRNA species. The Env precursor protein transcripts are targeted to the rough endoplasmic reticulum (ER) for translation, and the precursor protein is processed and transported via the cellular secretory pathway to the plasma membrane as mature gp120-gp41 envelope protein complexes. Other mRNA products are translated on cytoplasmic ribosomes to individual viral proteins or, in the case of Gag (group-associated antigen) and Gag-Pol (polymerase), precursor polyproteins. Full-length transcripts, destined to function as progeny viral genomic RNA, are also exported from the nucleus. Two copies of these are encapsidated by viral polyproteins after export.

Components of the progeny virion are targeted to the inner face of the plasma membrane for assembly and subsequent budding. After release, virions undergo a structural maturation process as the result of viral protease-catalyzed cleavage of Gag and Gag-Pol polyproteins into their individual components---matrix (MA), capsid (CA), nucleocapsid (NC), protease (PR), and p6 for Gag; RT and IN for Pol. During this maturation process, the viral particle's three-dimensional structure evolves from spherical to icosahedral, and the virion acquires the capacity to infect.

HIV-1 Viral protein R (Vpr): Structure, Functions, and Cellular Effects

HIV-1 Vpr is a 96 amino acid-long HIV-1 accessory gene product with an approximate molecular mass of 14kD and is produced on cytoplasmic ribosomes from singly-spliced viral mRNA (32-34). The Roques laboratory performed much of the pioneering work on elucidating Vpr's three-dimensional structure, a formidable task because of Vpr's small size and high propensity for self-aggregation in water. This laboratory's nuclear magnetic resonance (NMR) data, first on recombinant amino (N)-terminal and carboxyl (C)-terminal Vpr peptides and later on full-length synthetic Vpr, suggested that Vpr possesses a hydrophobic core surrounded by three alpha-helices. On either side of these helices, Vpr's N-and C-termini display little inherent structure and high degrees of flexibility (35-42). The arginine-rich, highly basic C-terminus may represent a protein transduction domain (PTD): virion-free, extracellular Vpr is frequently found in HIV patients' sera and cerebrospinal fluid (CSF) and has been reported to penetrate cell membranes (43-47). This transduction ability has been

suggested to play a role in HIV-1 pathogenic processes such as lymphocyte depletion via "bystander" cell death induction and the CNS neuron depletion of HIV-1-associated dementia. Vpr amino acid residues 17-29 and 35-46 form the first two predicted helices and are separated by a beta-turn; the third, formed from amino acids 53-78, possesses a central, 22 amino acid-long isoleucine/leucine-rich region that endows this helix with a leucine zipper-like structure (35-42). The structural domains of Vpr appear to be important for specific protein-protein interactions that modulate Vpr's functions and cellular effects.

Besides pre-mitotic host cell cycle arrest induction, a number of HIV-1 life cycle functions have been reported for Vpr, and the biological significance of most is associated with the proliferation status or capacity of the infected cell [reviewed in (24, 48, 49)]. These reported functions include: reverse transcription initiation involvement (50), limiting the mutation rate of HIV-1 DNA synthesis (51, 52), facilitating the nuclear import of the viral PIC (53), increasing viral replication by increasing transcription from the HIV-1 LTR (9, 13, 14), and affecting host cell survival and lifespan (3, 15, 22, 23, 59-73).

HIV-1 reverse transcription initiation requires a lysine transfer RNA ($tRNA^{lys}$) primer, and Stark and Hay proposed that Vpr has a role in this initiation through an interaction with lysine-tRNA synthetase that requires Vpr's N-terminal domains. This interaction has been suggested to prevent lysine-tRNA synthetase-mediated amino acylation of $tRNA^{lys}$, allowing primer targeting to and incorporation into the virion. These investigators speculated that, since deacylated tRNAs are less abundant in translationally inactive cells, this Vpr function might be more important in resting

lymphocytes and terminally-differentiated macrophages, cell types in which Vpr appears to be critical for viral replication (50).

Vpr's proposed ability to control the mutation rate associated with the error-prone reverse transcription process occurs via an interaction with a cellular uracil DNA glycosylase (UNG2) thereby preventing uracil incorporation into proviral DNA. Vpr is incorporated into virions at relatively high levels thereby allowing Vpr to function as a viral immediate early protein, and the Vpr-UNG2 interaction has been proposed to be the mechanism for inclusion of enzymatically active UNG2 into virions. The UNG2 interaction function of Vpr also appears to be more important in non- or slowly-proliferating cells: a Vpr mutant incapable of targeting UNG2 to the virion severely limited viral replication efficiency in primary monocyte-derived macrophages but had no effect on replication in primary blood mononuclear cells (PBMCs). A tryptophan residue within Vpr's third helix, Trp^{54}, appeared to be critical for its ability to interact with UNG2 (51, 52).

Another Vpr function in the viral life cycle particularly important in non-replicating cells is its contribution to PIC transport across the nuclear membrane. Since these cells do not undergo mitosis-associated nuclear envelope breakdown, a mechanism for PIC nuclear translocation is essential for productive infection. Vpr-binding partner interactions that have been suggested to be significant in the PIC translocation process are those with other viral components of the PIC, *e.g.* IN and viral DNA, and cellular trafficking proteins, *e.g.* (a) nucleoporin(s), importin-alpha, and a novel Vpr-interacting protein (RIP). The Vpr domain requirements for its PIC import function are nebulous, but its amino terminal regions appear to be particularly important for the few interactions

that have been elucidated. However, a nuclear export role associated with its third helix has also been suggested for Vpr (53-55).

In addition to indirectly increasing proviral transcription by arresting the cell cycle at a stage in which this process is most active, Vpr has been proposed to play (a) direct role(s) in the transcriptional activation process. Direct interactions of Vpr with Tat, the HIV-1 transactivator, and with an array of cellular transcription factors and transcriptional co-activators have been reported to affect both proviral and cellular gene transcription. Vpr-interacting cellular transcription components include Sp1, p300, transcription factor IIB (TFIIB), the glucocorticoid receptor (GR), and cyclin T1. Vpr's transcriptional activation ability is, however, controversial. At best, Vpr can be characterized as a weak LTR transactivator; most, if not all, of the observed experimental effects are probably attributable to Vpr's ability to arrest the cell cycle (9, 11-14, 56-58).

Vpr's effects on host cell survival have been studied extensively, but the nature and significance of these effects remain unclear. One broad unresolved question is whether Vpr promotes apoptosis, cell survival, or both. As discussed below, experimental evidence of Vpr's pro-apoptotic effects is substantial, but the mechanism(s) responsible and the biological relevance of Vpr-mediated apoptosis have not been established. Early investigations of Vpr's cell cycle arrest effects led to speculation that these effects increased the longevity of infected cells: He *et al.* suggested that cell cycle arrest might maximize virion production by suppressing the cellular apoptotic response and increasing viral persistence. Studies from a number of groups suggested that this might indeed be the case (3); and later, in attempts to explain the apparent contradiction of Vpr's abilities to promote both cell survival and apoptosis, two groups demonstrated

conditions under which Vpr might have opposite effects on cell survival. Ayyavoo *et al.* provided evidence that, in combination with initiation of T-cell receptor (TCR) signaling, Vpr blocked NFkappaB activities by inducing its inhibitor, IkappaB. Among the blocked activities were apoptosis, T cell activation, and NFkappaB-dependent cytokine production. Without concurrent TCR signaling, however, Vpr induced apoptosis (23). Conti *et al.* provided evidence that Vpr's influence on apoptotic decision-making might depend on the Vpr dose and infection stage of the cell: higher Vpr levels and later infection stages favored apoptosis, while survival was supported by lower expression at early times post-infection (15, 22). While suggesting that Vpr may indeed have diametrically-opposed effects on cell survival, the mechanistic orchestration and coordination of these functions and how these give rise to a selective viral advantage remain undefined.

Another enigma in the field of Vpr-mediated apoptosis is the vast range of mechanisms proposed for this process. Which, if any, occur during HIV-1 infection is impossible to decipher at this juncture. In two Stewart *et al.* studies, the Chen laboratory was first to demonstrate that caspase-dependent apoptosis followed G_2/M arrest in a variety of Vpr-expressing human cell lines (60, 61). The Kroemer group has since proposed a caspase-independent mechanism in which Vpr is directly toxic to mitochondria via association with the adenine nucleotide translocator (ANT) component of the mitochondrial permeability transition pore (MPTP) (62-65). Yuan *et al.* demonstrated that Vpr's ability to induce apoptosis might be Wee1-dependent as Vpr-induced apoptosis was suppressed by Wee1 over-expression in HeLa cells (66). In contrast to earlier Chen laboratory findings, Nishizawa *et al.* provided substantial

evidence that Vpr's ability to induce apoptosis was independent of its ability to arrest the cell cycle at G_2/M. Three isoleucine and leucine residues within Vpr's third helix were necessary for apoptosis induction in their experiments (69). Watanabe *et al.*'s findings suggested that centrosomal duplication abnormalities were one source of Vpr-induced apoptosis. This group concluded that these Vpr-induced abnormalities, occurring in G_2 phase of the cell cycle, triggered aberrant mitotic spindle formation that ultimately prevented normal nuclear and cell division. Apoptosis was the invariable consequence (70). Chang *et al.* provided evidence that anti-apoptotic members of the Bcl-2 protein family can suppress Vpr's apoptotic effects (71), and Zimmerman *et al.* have recently proposed that Vpr's ability to initiate apoptosis is a product of its ability to activate DNA damage and repair signaling pathways (72). While this list of findings is far from being an all-inclusive overview of the Vpr-mediated apoptosis field, the complications involved in assessing biological relevance of the findings are apparent.

The general question of biological relevance is the third and most important unresolved question in the field. While a minor role for Vpr might be conceded, the consensus among researchers is that HIV-1-mediated apoptosis is primarily an Env-mediated process, and some investigators refuse to concede even a minor role for Vpr in HIV-1-associated host cell apoptosis. Glynn *et al.*'s experiments using HIV-1-infected H9 cells, human $CD4^+$ T cells, demonstrated that the resulting cellular apoptosis, as well as being IL-1beta converting enzyme (ICE) protease-dependent, displayed no cell cycle phase specificity. This group's argument was that, if Vpr-mediated apoptosis were biologically significant, a correlation between the cell cycle stage of H9 cell apoptosis and the stage of Vpr-mediated arrest, G_2/M, should have been observed (73).

VPR-INDUCED HOST G_2/M CELL CYCLE ARREST

That Vpr causes G_2/M cell cycle arrest is its best-studied and most widely accepted function. Levy *et al.* provided the first substantive evidence that Vpr might function to prevent host cell proliferation, demonstrating that Vpr expression correlated with HIV-1 transfection-blocked proliferation of three human sarcoma cell lines. Vpr expression also correlated with induction of cellular differentiation in at least one of these, the TE671 rhabdomyosarcoma cell line (2). Rogel *et al.* provided the first evidence suggesting that Vpr-mediated cell cycle arrest is ultimately the result of Cdc2/Cdk1 inhibition (1). This group demonstrated that Vpr expression caused cell cycle profile redistribution; Vpr-expressing cells accumulated with *4N* DNA content suggestive of post-DNA replication cell cycle arrest. Separate subsequent studies from three different groups formally ruled out the possibility that these abnormal profiles had been the result of cytotoxic, rather than cycle-arresting, effects of Vpr. First, Jowett *et al.* reported that HIV-1-infected $CD4^+$ T lymphocytes and T lymphoblastoid cell lines accumulated in G_2 or early mitosis and demonstrated that Vpr was necessary and sufficient for this effect (5). One month later, both He *et al.* and Re *et al.* published results that confirmed accumulation of inactive Cdc2/Cdk1 in Vpr-arrested cells (3, 4). A decade and dozens of publications on Vpr's cell cycle effects later, inactivation of Cdc2/Cdk1 is the only mechanistic aspect of Vpr-induced G_2/M arrest uniformly accepted by researchers in this field.

Eukaryotic Cell Cycle Regulation Overview

This overview is provided to facilitate understanding of subsequent sections. Because the *Schizosaccharomyces pombe* (*S. pombe*) system has proven to be extremely valuable in elucidating the basis of Vpr's cell cycle effects, information on both fission yeast and mammalian cell cycle progression regulation is provided. Excellent, detailed reviews covering cell cycle regulation are widely available [examples include (26-31)].

Cyclin-dependent kinases (Cdks) are the master regulators of cell cycle transitions. Cell cycle regulation in mammalian cells is effected through multiple Cdks in association with different cyclins. Active mammalian G_1 cyclin-Cdk complexes, comprised of cyclin D family members with Cdk4 or Cdk6, are inactivated to allow Cdk2-mediated transition to and through S phase. Two associated cyclin partners, cyclin E and cyclin A, mediate the activity and functions of Cdk2. Cdk1 and a B-cyclin family member, particularly cyclin B1, are the primary components of the mammalian mitosis-promoting factor that regulates the transition from G_2 into prophase and progression through mitosis. In fission yeast, however, Cdc2, a Cdk1 homologue, regulates all cell cycle transitions; a variety of associated cyclins dictate cell cycle phase and function specificity.

Since Cdks are generally stable molecules and their abundance within the cell is usually non-limiting, their activities are generally assumed to be predominately controlled by post-translational mechanisms. One of these mechanisms, as their Cdk designation implies, is the association with a cyclin binding partner, an association necessary, but not sufficient, for enzymatic activation. Unlike mammalian Cdk1, *S. pombe* Cdc2 activity is required for both the duplication and segregation of fission yeast

chromosomal DNA. Four cyclins with cell cycle regulatory functions have been identified in association with fission yeast Cdc2: Cig1, Cig2, Puc1, and Cdc13. Cig2 is the major S-phase cyclin that, in a similar manner to mammalian cyclin E, accumulates late in G_1 and degrades prior to the onset of G2. Cdc13 is the *S. pombe* mitosis-promoting cyclin partner of Cdc2.

Activating phosphorylation by Cdk-activating kinases (CAKs) is a second major mechanism for positive regulation of Cdks and is usually required for full Cdk enzymatic activity. A common structural feature of Cdks is a "T loop", and CAK-mediated phosphorylation of a threonine residue within this region, *e.g.* Cdk2's Thr^{169}, is usually required for maximal Cdk enzymatic activity. The core components of the mammalian Cdk1 CAK comprise a Cdk-cyclin complex themselves, Cdk7- cyclin H; these CAK components also participate in RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. The fission yeast CAK has similar functions in both Cdc2 "T loop" threonine and RNA polymerase II CTD phosphorylation.

Inhibitory phosphorylation of Cdc2/Cdk1 is a major mechanism for preventing uncontrolled cell proliferation. The fission yeast Mik1 and Wee1 kinases and their homologues in other eukaryotic cells phosphorylate the residues equivalent to Cdc2's Thr^{14} and Tyr^{15}, modifications that prevent mitotic Cdk activity. While these residues are adjacent to the Cdk's ATP-binding site, the molecular mechanism for this method of inhibition has not been firmly established. The fission yeast Cdc25 dual-specificity phosphatase and its multiple homologues in higher eukaryotes, Cdc25A/B/C, are responsible for regulating mitotic entry by dephosphorylating the Cdc2 Thr^{14} and Tyr^{15} equivalents. Cdc25C is generally considered to be the homologue primarily responsible

for Cdk1 dephosphorylation; however, since Cdc25C knockout mice suffer no apparent mitotic abnormalities, functional redundancy and/or overlap among the homologues is likely.

Binding of Cdks, with or without an associated cyclin, to endogenous cyclin-dependent kinase inhibitors (CKIs) is a second principal mechanism for directly preventing CDK activation. This mechanism is most prominent during G_1, but a few CKIs may also play a role in regulating steps leading to mitotic entry. Rum1, the only identified fission yeast CKI, associates with Cdc2 during exit from mitosis as Cdc13 is degraded. This association prevents significant Cdc2 activity during G_1. As cells approach the G_1/S boundary, phosphorylation-dependent Rum1 degradation occurs, lifting restrictions on Cig2-Cdc2 activity and allowing cell cycle progression through the G_1/S restriction point.

Vpr's cell cycle effects do not appear to result from altered cyclin B or Cdk1 expression levels; and direct interaction of Vpr with Cdk1 and cyclin B, alone or in complex, has never been demonstrated. The logical inference is that Vpr blocks mitotic entry and, therefore, cellular proliferation, by interacting with (a) Cdk1 regulator(s). Preventing uncontrolled proliferation of cells while meeting developmental and replacement demands requires that Cdks and their regulators be under strict controls. Since many factors affect the cellular decision to proliferate, these control mechanisms incorporate signals from the numerous pathways that monitor both extracellular and intracellular environmental conditions. The multitude and complexity of pathways regulating Cdc2/Cdk1 activity offer an abundance of potential mechanisms by which Vpr might induce cell cycle arrest.

A substantial amount of experimental evidence suggests that aberrant regulation of Wee1 and Cdc25C plays an essential role in Vpr-induced G2 arrest, and, like Cdc2/Cdk1, Wee1 and Cdc25 are themselves regulated temporally and spatially by intricate networks of transcriptional, translational, and post-translational mechanisms during cell cycle progression. All of the proposed models discussed in this thesis suggest that Vpr-mediated arrest occurs via pathways that regulate Wee1 and/or Cdc25.

Cell Cycle Checkpoint Activation Models of Vpr-Mediated Arrest

One point of contention in the study of Vpr's cell cycle effects is the role of cell cycle checkpoint signaling pathways in mediating these effects. Cell cycle checkpoints monitor the completion of intracellular events essential for cell proliferation, ensuring the appropriate temporal and spatial coordination of these events. Oversight of DNA maintenance and metabolism is a particularly important function of cell cycle checkpoints as the genome is especially susceptible to disastrous alterations resulting from a variety of stresses, including those associated with meeting the demand to proliferate [reviewed in (74-76)].

While checkpoint signaling pathways are far from being completely understood, the molecular signals that activate DNA-associated responses appear to be unusual DNA structures or complexes, *e.g.* double-stranded DNA breaks (DSBs) and single-stranded DNA (ssDNA) (77, 78). These may occur either in S phase in conjunction with stalled or collapsed replication forks as the result of stress during replication or in any phase of cell cycle progression in response to genotoxic events. In mammalian cells, two phosphatidylinositol-3 kinase (PI3K)-like kinases (PIKKs), ataxia telangiectasia-mutated

(ATM) and ATM and Rad3-related protein (ATR), are known upstream transducers of subsets of checkpoint-activating signals. DNA lesion specificity may play some role in their activation: ATR appears to be activated in response to ssDNA or DSBs processed into single-strand lesions, and ATM activation appears to occur when DNA DSBs are generated, particularly in response to gamma-irradiation. Some degree of functional overlap between ATR and ATM is probable, however.

Unlike the lipid targets of PI3K, ATR and ATM phosphorylate proteins, and their substrate specificities also overlap. In addition to proteins involved in cell cycle regulation, targets of these PIKKs are involved in processes such as chromatin remodeling at sites of DNA damage and DNA repair. Known ATR targets include: Chk1, Rad17, the H2A variant X histone (H2AX), BRCA1, Plk1, p53, 53BP1, and E2F. A single fission yeast PIKK, Rad3, appears to perform the relevant functions of both ATM and ATR [reviewed in (74-76)].

In fission yeast and vertebrate cells, the primary downstream effectors of activated G_2/M checkpoint-mediated arrest are Cdc25/Cdc25C and Wee1. Chk1 and Cds1/Chk2, downstream checkpoint kinases activated by ATR and/or ATM, catalyze inhibitory Ser^{216} phosphorylation of Cdc25/Cdc25C. In addition to directly inhibiting Cdc25C phosphatase activity, Cdc25C Ser^{216} phosphorylation creates a 14-3-3 protein binding site, facilitating Cdc25C-14-3-3 interactions. These interactions result in Cdc25C nuclear export and cytoplasmic sequestration, further inhibiting its ability to activate Cdc2/Cdk1. Chk1 also enhances Wee1's ability to maintain Cdc2 in an inactive state by phosphorylating Wee1 on Ser^{549}. 14-3-3 proteins are again implicated as playing a major role in the G_2/M checkpoint: Wee1 Ser^{549} phosphorylation results in its

association with 14-3-3 proteins, an association that has a positive regulatory effect on Wee1 [reviewed in (74, 75, 81)].

The mammalian p38 mitogen-activated protein kinase (MAPK) and homologous fission yeast Sty1 stress-activated protein kinase (SAPK) pathways have recently been demonstrated to function in a third, ATR- and ATM-independent, G_2/M checkpoint signaling pathway. In response to hyperosmotic stress, p38 and Sty1 activate MAPKAP kinase-2 (MK2) and Srk1, respectively, which subsequently phosphorylate Cdc25 homologues, inducing their binding to and cytoplasmic sequestration by 14-3-3 proteins. This occurs in a manner apparently identical to Chk1-mediated activities after DNA damage-induced Rad3 homologue activation. One group has suggested that MK2 be renamed Chk3, a suggestion supported by their observation that MK2 inactivation prevents a functional UV irradiation-induced G_2/M checkpoint activation response. The activating signal(s) for the SAPK-dependent G_2 checkpoint is/are unknown but may be stress-induced cellular alterations not directly involving DNA (79, 80).

Another aspects of DNA damage responses relevant to the ensuing discussion include the effects of treatment with methylxanthine derivatives. Methylxanthine derivatives, like pentoxifylline and caffeine, are pharmacological inhibitors of ATR and ATM. These effects are, however, non-specific; other biochemical activities, *e.g.* cyclic nucleotide phosphodiesterase (PDE) inhibitory activity, are associated with these agents. These have been employed in a number of studies attempting to elucidate the molecular mechanism(s) behind Vpr's ability to arrest the cell cycle. Methylxanthine derivatives selectively sensitize certain tumor cells, especially those that are p53-deficient, to radiation and chemotherapeutic agents. This effect, eventually attributed to their abilities

to inhibit ATR and ATM activities, was, until recently, thought to be the direct result of G_2/M checkpoint dysfunction. Mitotic entry prior to adequate DNA repair was believed to trigger cell death in these treated tumor cells. Recent data suggest, however, that methylxanthine treatment leads to inhibition of a PIKK-mediated step in the DNA repair by homologous recombination (HRR) process and that this, not reduced repair time, is responsible for the increased sensitivity seen with combination therapy [reviewed in (82)].

Summary of Relevant Data

The end result of ATR- and/or ATM-mediated G_2 checkpoint activation is a G_2 arrest characterized by Cdc25C inactivation and hyper-phosphorylation of Cdc2/Cdk1, similar to the arrest seen in Vpr-mediated arrest. This observation has led to tests of the hypothesis that Vpr mediates arrest by activating a G_2/M checkpoint signal transduction pathway, either by directly damaging cellular DNA or by mimicking an ATR- or ATM-activating signal. Early tests of this hypothesis were not supportive, however. Bartz *et al.* tested Vpr's ability to elicit G_2 arrest in cells lacking functional ATM (AT cells) and discovered that absence of this checkpoint-associated PIKK had no effect on Vpr's action. Additionally, re-replication of the cellular genome was not prevented in a sub-population of HIV-infected, Vpr-expressing Jurkat T cells: a significant percentage of cells infected with *vpr*$^+$, but not *vpr*$^-$, experimental HIV strains became tetraploid. The presence of both multi- and mono-nucleated sub-populations of these *8N vpr*$^+$ cells suggested that at least some had entered mitosis. Unlike DNA damage-induced arrest,

Vpr-induced arrest in HeLa cells appeared to be unaffected by treatment with methylxanthine derivatives in the Bartz *et al.* experiments (90).

Both Masuda *et al.* and Elder *et al.* tested the requirement for G_2/M cell cycle checkpoint-associated genes in Vpr's arrest of the *S. pombe* cell cycle. In these studies, Vpr was expressed in a number of checkpoint-deficient fission yeast strains, and none of the associated mutations reversed Vpr's anti-proliferative and arrest-inducing effects. The upstream checkpoint genes tested in these experiments included *rad1*, *rad3*, *rad9*, and *rad17*; the downstream effector kinase genes, *chk1* and *cds1*, were also mutated alone or in combination in these studies. Elder *et al.* did, however, demonstrate that treatment with 5mM pentoxifylline alleviated Vpr-induced arrest in wild-type fission yeast, and Masuda *et al.*'s data did suggest that proliferation of Vpr-expressing delta*chk1* or *rad1-1* fission yeast recovered to control levels at late time points post-Vpr expression (91-93).

Poon *et al.* were first to propose that, in human cells, Vpr does, indeed, arrest the cell cycle by activating a signaling pathway linking DNA damage recognition to cell cycle progression regulation. This group noted that Vpr-expressing cells and those treated with a DNA alkylating agent, nitrogen mustard (HN2), shared common features---both failed to enter mitosis following DNA replication and possessed inactive, hyper-phosphorylated Cdk1 (94). Poon *et al.* tested their proposal in HeLa cells and demonstrated that similarly abnormal cell cycle profiles resulted from Vpr expression and HN2 treatment and that these profiles correlated with similarly increased quantities of slower-migrating, presumably hyper-phosphorylated, CDK1 species on immunoblot. CDK1 isolated from both HN2-treated and Vpr-expressing HeLa cells possessed

substantially reduced enzymatic activity towards its physiological substrate histone H1. Poon *et al.* also demonstrated that methylxanthine treatment partially reversed the G_2 block caused by Vpr expression while completely reversing the HN2-mediated arrest. Poon *et al.* suggested a reasonable resolution for the inconsistency between their findings and those of the earlier Bartz *et al.* study. That group had withheld pentoxifylline treatment for 90 hours post-infection while cells were treated within 12 hours of infection in the later study. Poon *et al.* agreed that, in cells arrested by Vpr for more than three days, the arrest was not reversible by methylxanthine derivatives. Because Vpr's arrest function is an early post-infection event, the Poon *et al.* findings are probably more applicable (90, 94). Subsequently, in both Zhu *et al.* and Roshal *et al.*, the Planelles group demonstrated that caffeine also partially disrupted Vpr's ability to induce G_2 arrest in human cells (95, 96).

In Roshal *et al.* and, later, Zimmerman *et al.*, the Planelles laboratory pursued further characterization of the DNA-associated signaling pathway(s) potentially involved in Vpr's ability to induce cell cycle arrest (96, 97). Roshal *et al.* showed that, in addition to caffeine, LY294002, a PI3K and PIKK inhibitor, partially reversed the ability of a vpr^+ lentiviral vector to delay or arrest HeLa cell cycle progression. Approximately 65% of vpr^+ virus-infected HeLa cells were in G_2/M at 36 hours post-infection; caffeine or LY294002 treatment reduced this percentage and increased the G_1 percentage by 20-25%. These data, along with those of Bartz *et al.*'s demonstration of the ATM-independence of Vpr's cell cycle effects, led to the hypothesis that Vpr expression results in the activation of ATR. Neither caffeine nor LY294002 completely reversed Vpr's effects as treated control cells infected with a vpr^- lentivirus possessed G_2/M populations

significantly different from those of Vpr-expressing cells: caffeine- and LY294002-treated control G_2/M cells represented 17.3% and 25.5% of the total populations, respectively, while the comparable *vpr*$^+$ populations were both greater than 40% (96).

Roshal *et al.* first tested the relationship between ATR activation and Vpr's cell cycle effects through the inducible expression of a dominant-negative ATR (termed ATRkd for kinase-dead ATR) in two cell lines, U2OS/ATRkd and GM847-ATRkd. These investigators used the lentiviral expression system employed in the pharmacological inhibitor experiments to compare the cell cycle expression profiles of mock-infected; control, *vpr*$^-$ lentivirus-infected; and *vpr*$^+$ lentivirus-infected cells under ATRkd-induced and non-induced conditions. In the absence of ATRkd induction, the G_2/M population of control virus-infected U2OS/ATRkd cells was, indeed, small compared to that of Vpr-expressing cells, 4% versus 47%; and upon ATRkd induction, this difference was dramatically reduced to 14% versus 21%. In a separate experiment, ATRkd induction was also demonstrated to suppress Vpr's purported ability to transactivate an HIV LTR-containing promoter, a function reported to correlate with its ability to induce cell cycle arrest (96).

Beyond a simple comparison of G_2/M population percentages, the flow cytometric histograms from the ATRkd cell cycle experiments may indicate other important differences among the populations. For example, defective lentiviral infection in the absence of Vpr expression appeared to cause an increased percentage of cells in S-phase. In the presence of ATRkd, this S-phase percentage was virtually equivalent to that seen with Vpr expression, but the distribution patterns of the S-phase sub-populations were different. The S-phase Vpr-expressing cells conformed to a bell-shaped pattern like

that of the mock-infected, ATRkd-induced cells while the comparable *vpr*$^-$ control virus population appeared to possess predominately early S-phase cells. The histogram pattern most important to the subject of this thesis was that of the G_2/M population of Vpr-expressing cells in the absence of ATRkd induction. This pattern covered a broad range of propidium iodide staining intensities suggestive of a significant percentage of cells with greater than *4N* DNA content, and ATRkd expression appeared to correct this abnormality.

Roshal *et al.* tested the effects of small, interfering RNA (siRNA) targeted to either ATR or Chk1 mRNA on Vpr-mediated arrest. A 70% decrease in ATR expression by siRNA knockdown substantially reduced the percentage of Vpr-expressing cells in G_2/M from greater than 60% to 36% and, like ATRkd, prevented the broad-range propidium iodide staining intensities described above. Also like ATRkd expression, ATR siRNA did not completely reverse Vpr's cell cycle effects as control cells in G_2/M represented only 9% of the total. Unlike ATRkd expression, ATR siRNA had no effect on the cell cycle distribution profile of these control, *vpr*$^-$ virus-infected cells; the large S-phase (~50%) population was maintained whether ATR-specific, irrelevant, or no siRNA was introduced (96).

Roshal *et al.*'s remaining experiments centered on elucidating the effects of Vpr expression on an important ATR substrate, Chk1. Their Chk1 siRNA reduced its expression by 90% in HeLa cells. The associated G_2/M percentage reduction in Vpr-expressing cells was, again, approximately 20-25%---from 65-70% to 45%. The corresponding mock-infected G_2/M population represented 12% of the total, and no *vpr*$^-$ virus-infected control population was employed. Vpr expression induced Chk1 Ser^{345}

phosphorylation similar to that occurring after ATR activation, and caffeine suppressed this Chk1 phosphorylation by approximately 60%, consistent with its ability to inhibit ATR. Also, treatment of *vpr*$^+$ lentivirus-infected HeLa cells with UCN-01, a non-specific Chk1 inhibitor, resulted in a 75% to 40% G_2/M cell population shift (96).

Zimmerman *et al.* tested Vpr expression effects on three additional known ATR substrates--- Rad17, BRCA1, and H2AX. Rad17 is thought to replace Rfc1 in the pentameric replication factor C "clamp loader complex" to form a complex with analogous function in DNA damage and repair signaling. Available data suggest that Rad9, Rad1, and Hus1 form a "9-1-1 complex" possessing a DNA damage response function analogous to that of the proliferating cell nuclear antigen (PCNA) homotrimeric "sliding clamp" in DNA replication. Zou *et al.*'s data suggest that ATR, Rad17 and the 9-1-1 complex co-localize in a checkpoint activation step, and both Rad17 and the 9-1-1 complex appear essential for ATR-mediated cell cycle arrest. Rad17 is probably ATR's first target during checkpoint signaling; Hus1, while not a direct ATR target, seems necessary for ATR's ability to phosphorylate Rad17 [(83), reviewed in (74, 75, 84)]. ATR-mediated cell cycle arrest signaling events seem to require Hus1, but ATR-mediated DNA repair signaling processes do not (85).

Rogakou *et al.* demonstrated that the variant X form of histone 2A becomes phosphorylated in response to DNA DSBs (86); Burma *et al.* and Ward *et al.* subsequently demonstrated ATM- and ATR-mediated H2AX phosphorylation, respectively (85, 87). Phosphorylated H2AX accumulates in nuclear foci presumably to recruit components of DNA damage recognition, signaling, and repair complexes (88). BRCA1 has been demonstrated to be functionally important in a number of cellular

processes including centrosome duplication, cell cycle checkpoint signaling, DNA repair, and apoptosis induction. BRCA1 is a substrate for a number of kinases, including ATR; therefore, BRCA1 may integrate signals from a number of cellular pathways to coordinate cell cycle progression-related processes. Like H2AX, phosphorylated BRCA1 accumulates in a characteristic multifocal staining pattern (88, 89).

Using the same *vpr*$^{+}$ versus *vpr*$^{-}$ lentivirus-infected HeLa cell system of Roshal *et al.*, Zimmerman *et al.* demonstrated that an 85% reduction of Rad17 by siRNA resulted in a 70% to 40% G_2/M cell population shift. In contrast to the large S-phase populations of Roshal *et al.*'s experiments, the *vpr*$^{-}$ virus-infected cells in these experiments possessed cell cycle distribution profiles virtually identical to the mock-infected controls; in the presence of Rad17 siRNA, the G_2/M populations of both were slightly less than 20% of the total populations. Immunofluorescence studies demonstrated that Vpr expression also resulted in focal intranuclear deposition of both BRCA1 and phosphorylated H2AX in HeLa cells, and this localization result was confirmed for H2AX in CD4^{+} thymocytes infected with a full-length experimental viral clone, HIV-1_{NL4-3}. In a subsequent study, the group provided data suggestive of Vpr expression-induced BRCA1 phosphorylation, presumably the result of ATR activation (97).

Since ATR's abilities to phosphorylate Rad17 and to trigger cell cycle arrest require Hus1, Zimmerman *et al.* tested the requirement for Hus1 in Vpr-mediated G_2/M arrest by comparing the cell cycle effects of *vpr*$^{+}$ lentiviral infection in Hus1$^{-/-}$ p21$^{-/-}$ mouse embryonic fibroblasts (MEFs) to those in p21$^{-/-}$ MEFs. Mock-infected and *vpr*$^{-}$ virus-infected controls were included in these experiments. The presence of Hus1 was associated with a G_2/M arrest in *vpr*$^{+}$ virus-infected cells: flow cytometry determined that

greater than 70% of these were in G_2/M, and the histogram provided displayed the broad-range propidium iodide staining observed in similar Roshal *et al.* experiments. The cycle distribution profile of Vpr-expressing MEFs in the absence of Hus1 was virtually identical to that of the *vpr*$^-$ virus-infected controls. Hus1 appeared to influence the distribution profile of this control population, however: its G_2/M percentage rose from 11% to 30% in the absence of Hus1 with a corresponding opposite shift in S-phase percentage (97).

Vpr-mediated G_2/M Cell Cycle Checkpoint Activation Models

Poon *et al.* generated the first model invoking participation of a DNA damage signaling pathway in Vpr-induced G_2/M arrest based on the similarities of this arrest with that induced by HN2 treatment. Little was known about the cellular components of DNA damage-associated checkpoint pathways at that time; the only detail that Poon *et al.* could add to the model was the possible involvement of a previously demonstrated Vpr-uracil DNA glycosylase (UNG) association in the arrest mechanism (94).

Roshal *et al.* speculated that Vpr-induced nuclear envelope herniations result in chromatin structural changes that ultimately cause replication arrest and ATR activation. Cell cycle arrest in this model was attributed to Chk1-mediated inhibition of Cdc25C and/or activation of Wee1 with subsequent failure to activate Cdk1/Cdc2 (96). This model incorporated their data with those of de Noronha *et al.* who previously demonstrated that Vpr over-expression caused formation and intermittent rupture of lamin-associated nuclear envelope herniations. de Noronha *et al.* had suggested that the ruptured herniations might result in interruption of DNA replication or aberrant sub-

cellular compartmentalization of Wee1 and Cdc25C, either of which might cause cell cycle arrest (98).

The Roshal *et al.* model was abandoned in the Zimmerman *et al.* discussion. Instead, this follow-up focused on possible molecular mechanisms of Vpr-mediated ATR activation and attempted to integrate its findings with a model recently offered by the Emerman group in which Vpr-Cdc25C direct association was proposed to participate in G_2 arrest induction (99). Zimmerman *et al.*'s premise was that the actual Vpr-mediated arrest mechanism should be or mimic one evoked by ssDNA or processed DSB lesions, but not one activated by unprocessed DSBs. Among the possible mechanisms identified were: (1) Vpr might possess nuclease activity and directly generate ATR-activating lesions, (2) Vpr might indirectly cause such lesions via interactions with an endogenous nuclease or cellular component, *e.g.* chromatin, capable of disrupting replication fork progression, or (3) Vpr-DNA interactions might cause or mimic DNA lesions or stalled replication forks. The potential for direct Vpr-ATR interactions was also suggested; however, the group was unable to demonstrate a Vpr-ATR physical association (97). The reasons for consolidating their model with that of Goh *et al.* was not well developed; and neither of the integration options offered were intellectually appealing. These suggestions that Vpr's cell cycle effects occur by multiple mechanisms or that signals from downstream checkpoint pathway components such as Cdc25C may be transmitted to DNA damage recognition pathway components by as-yet unappreciated means (97). More likely is the option that either or both models are incorrect.

Discussion of Data and Models

Among the data cited as evidence of G_2/M cell cycle checkpoint activation as a mechanism for Vpr-mediated arrest are those demonstrating that methylxanthine derivatives partially relieved Vpr-mediated cell cycle arrest in mammalian cells (94, 96). Since loss of functional ATM had no effect on Vpr-mediated arrest (90, 97), these findings suggest that a signal emanating from ATR may be responsible for Vpr's cell cycle effects. However, the scope of cellular enzymes affected by methylxanthine derivatives is not limited to ATR and ATM; and cellular effects of methylxanthine-mediated ATR and ATM inhibition appear to be more a product of recombinational DNA repair, rather than cell cycle checkpoint, inhibition. Also, while effects of methylxanthine derivatives on the newly identified p38/Sty1 checkpoint pathway have not been described, the possibility of such effects cannot be disregarded.

More importantly, the fission yeast methylxanthine treatment results directly challenge the ATR activation model for Vpr-induced arrest. The methylxanthine-mediated suppression of Vpr's effects was distinctly more profound in *S. pombe* than in human cells: pentoxifylline totally reversed Vpr's ability to induce cell cycle arrest, as well as the morphological defects and cytotoxicity caused by Vpr expression, in fission yeast (68, 93). However, loss of function mutation of *rad3*, the only identified fission yeast checkpoint PIKK, had no suppressive effect on Vpr-mediated arrest. Neither did loss of function mutations in a host of upstream and downstream G_2/M checkpoint genes to include *rad1*, *rad9*, *rad17*, *chk1*, and *cds1*, as well as a *chk1cds1* double mutation (91, 92). Vpr-mediated arrest in fission yeast is independent of G_2/M checkpoint activation.

The degree of methylxanthine-mediated reversal is debatable. Poon *et al.* speculated that, had their methods allowed for transient, rather than constitutive, Vpr expression, methylxanthine reversal of Vpr-induced arrest might have been equaled that observed on HN2-mediated arrest (94). Also, Roshal et al. interpreted their caffeine suppression data as indicative of complete reversal Vpr-mediated arrest even though the cell cycle profiles of caffeine-treated control and Vpr-expressing cell populations were substantially different (96). Even if complete methylxanthine treatment were to completely suppress Vpr's effects in human cells, the likelihood that this suppression would occur by an entirely different mechanism than that occurring in fission yeast is remote. However, Roshal *et al.* argued that different mechanisms of action with identical outcomes are, indeed, responsible: fundamental functional differences between Rad3 and ATR may explain the discrepancies between the two experimental systems. For example, ATR has a more extensive role than Rad3 in genome surveillance, functioning as a crucial sensor of incomplete DNA replication in the absence of exogenous genotoxins while Rad3 has no such role. The Roshal *et al.* argument followed that Vpr utilizes an alternate pathway to arrest the fission yeast cell cycle with the implication that this pathway is at least as sensitive to methylxanthine treatment as the ATR pathway (96).

Roshal *et al.* also used their interpretation of the caffeine suppression data to support their belief that the residual Vpr-induced arrest observed after siRNA-mediated ATR and Chk1 reduction was due to incomplete protein knockout and not a second pathway of action (96). Zhao and Elder countered that the ATR activation observed in the Planelles laboratory's experiments might be the result of either the lentiviral vector integration process or the activation of the protein phosphatase 2A (PP2A)-dependent

pathway actually used by Vpr to arrest of the cell cycle (100). (The Zhao and Elder model (93, 100) and that of Masuda *et al.* (91), both of which espouse checkpoint-independent mechanisms will be discussed in the next section. PP2A is a central mediator of Vpr-induced arrest in the Zhao and Elder.) All suggestions as to how ATR was activated in the Planelles laboratory's experiments are little more than speculative. Zhao and Elder's suggestion that these may be due to the integration process of the experimental vectors is possible; but whether or not retroviral integration consistently triggers ATR activation is an unresolved question, and the majority of the Planelles groups' experiments included *vpr*$^-$ lentiviral vector controls which did not appear to activate ATR. Interestingly, however, Nunnari *et al.* have provided evidence that caffeine's inhibitory effect on HIV-1 replication occurs via inhibition of proviral integration, presumably through negative effects on PIKK activity (101). If these and the Planelles findings are both accurate, questions arise involving possible roles for Vpr in proviral integration.

Zhao and Elder's suggestion that ATR activation may have resulted through a PP2A-mediated process is also possible, but no PP2A pathway regulating ATR (or Rad3) activation has yet been demonstrated. The Zimmerman *et al.* list of molecular mechanisms by which Vpr might activate ATR appears all-inclusive based on current models of DNA damage and cell cycle checkpoint signaling, but little or no evidence demonstrating that any of these is relevant to Vpr-mediated arrest is available (97).

While the data of both Planelles group experiments strongly suggested that ATR was activated in the presence of ATR, the mechanism for and biological consequences of this activation were not conclusively settled. Is ATR activation totally responsible for

Vpr's ability to arrest the cell cycle? Probably not; no experimental manipulation of ATR expression levels, or those of Chk1 or Rad17 targets, resulted in complete ablation of Vpr-mediated arrest (96, 97). More significantly, this interpretation of ATR's role ignores a wealth of data generated in the field of Vpr research. Is ATR activation partially responsible for Vpr's ability to arrest the cell cycle? This is more likely; but available data, *e.g.* those from methylxanthine treatment experiments, currently favor an interpretation that the mechanism of Vpr arrest in *S. pombe* and human cells is similar. The Planelles laboratory's interpretation precludes such similarity. Until the molecular mechanism of ATR activation is demonstrated, the most likely explanation is that this activation occurs via cross talk with a pathway directly affected by Vpr. Does ATR activation play a part in maintaining, if not initiating, Vpr-mediated arrest? This is quite possible, if not probable. In *S. pombe*, Vpr-mediated proliferation blocks in a delta*chk1* mutant strain were eventually overcome (91), suggestive of an arrest maintenance role for Rad3.

Frequently overlooked is the Bartz *et al.* demonstration that tetraploidy occurs in a significant percentage of cultured T cells as a consequence of vpr^+ HIV-1 infection (90). Therefore, G_2/M arrest may not be a universal cell cycle progression response to Vpr expression. Other data (discussed later) from both fission yeast and human cell systems emphasize the potential importance of the ability of some Vpr-expressing cells to re-replicate their genomes with and/or without entering mitosis. ATR activation might influence DNA re-replication in Vpr-expressing cells: ATR inhibition by ATRkd expression or siRNA appeared to reverse Vpr-mediated hyper-ploidy (96, Figure 3A and Figure 4B) as did siRNA-mediated inhibition of its target, Rad17 (97, Figure 1B). Since

inhibition of Chk1 by siRNA reduced Vpr-mediated cell cycle arrest without seeming to affect hyper-ploidy (96, Figure 7B), Vpr's aberrant DNA re-replication effects appear to result from an ATR-dependent process that is independent of ATR's cell cycle checkpoint functions.

Vpr Effects in *S. pombe*: PP2A- and Wee1-dependent Cell Cycle Arrest

Summary of Relevant Data

Zhao *et al.* were first to establish the utility of *S. pombe* as a model system for studying the cellular effects of Vpr. By expressing *vpr* from a thiamine-repressible promoter, this group demonstrated that Vpr induced changes in fission yeast similar to those previously observed in mammalian cells. Arrested or delayed cell cycle progression, correlating with Cdc2/Cdkl inhibition, was among these similarities (102). Zhang *et al.* subsequently published additional data on fission yeast cell cycle defects resulting from *vpr* expression, confirming many of Zhao *et al.*'s findings (103). Both groups demonstrated that Vpr expression induced morphological abnormalities within fission yeast cells, both provided flow cytometric data demonstrating Vpr's ability to arrest the fission yeast cell cycle, and the flow cytometric data of both showed that sub-populations of Vpr-expressing cells over-replicate DNA in the absence of cell division. The results of both studies also suggested that Vpr expression caused Cdc2 inactivity: Zhao *et al.* demonstrated by immunoblot that Vpr expression led to a decrease in the presence of the active, hypophosphorylated form of Cdc2; and Zhang *et al.*'s biochemical histone H1 kinase assays demonstrated Vpr-mediated suppression of Cdc2 enzymatic activity. One significant difference in the results of these early Vpr-fission yeast studies

was the cell cycle stage(s) at which Vpr induced arrest. Zhao *et al.* concluded that Vpr arrested fission yeast at G_2, but the data of Zhang *et al.* suggested G_1 and G_2 arrest as well as an impaired ability of Vpr-expressing cells to transit S phase. The latter group offered the plausible explanation that, since Cdc2 regulates initiation of DNA synthesis as well as mitotic entry in fission yeast, cell cycle delays at multiple stages should be expected. Neither group, however, explained the cell sub-populations possessing DNA content greater than that expected for cells arrested in G_2 (102, 103).

Masuda *et al.* later identified *S. pombe* cellular factors that mediate Vpr's cell cycle effects by comparing these effects in wild-type fission yeast to those in strains with mitotic entry defects. Vpr expression induced a *cdc phenotype* typical of G_2-arrested fission yeast in the wild-type and eight of 13 mutated strains and arrested proliferation in all 14 strains tested. The strains in which the Vpr-induced *cdc phenotype* was suppressed included delta*ppa2,* delta*rad24,* and three strains with compromised Wee1 function (*cdc2-1w, wee1-50* grown at the non-permissive temperature, and delta*wee1*). These data demonstrated Vpr interactions with *ppa2, rad24,* and *wee1*, encoding the major fission yeast protein phosphatase 2A (PP2A) catalytic subunit, one of two *S. pombe* 14-3-3 protein homologues, and the Wee1 tyrosine kinase, respectively. In addition to the cell cycle checkpoint-associated proteins discussed in the previous section, cell cycle regulators with no demonstrable role in Vpr-induced cell cycle effects in this study were: Cdc25; Nim1, an upstream regulator of Wee1; Mik1, a Wee1-like Cdc2-inhibiting kinase; and Ppa1, the second fission yeast PP2A catalytic subunit. While not mentioned by the authors, the proliferation of the *cdc2-3w* strain appeared to return to near-control levels at late experimental time points (>60 hours post-*vpr* induction). Since cdc2-3w is

partially Cdc25-independent, attributable to either Cdc25-independent Tyr^{15} dephosphorylation or to its retention of some kinase activity in the face of wild-type Tyr^{15} phosphorylation levels, the proliferation data may suggest a role for Cdc25 in maintaining, but not establishing, the Vpr-mediated arrest (91).

A potentially important Masuda *et al.* observation was that cell proliferation was similarly inhibited whether or not the *cdc phenotype* was suppressed. These investigators reasonably expected that, along with "abrogation" of G2 arrest in the Weel-, Rad24-, and Ppa2-deficient strains, there should be an associated increase in cell proliferation to that of wild type levels. From starting concentrations of 10^5 cells/ml, proliferation ceased at cell densities of $<10^7$/ml by 15-20 hours post-*vpr* induction in all 14 strains tested, while control cells entered stationary phase 15-20 hours later at $>10^8$ cells/ml (91).

In two subsequent *S. pombe* studies, Elder *et al.* confirmed and expanded upon most of the Masuda *et al.* findings. In the first of these, in addition to the checkpoint protein findings discussed previously, Elder *et al.* tested the importance of Cdc2 Tyr^{15} phosphorylation and the relative contributions of Wee1 and Cdc25 to this arrest. Vpr expression had no effect on the cell cycle distribution profile of the PR714 *S. pombe* strain in which Cdc2 is constitutively active as the result of a Tyr^{15} to Phe^{15} mutation. This observation strongly suggested a requirement for Tyr^{15} in Vpr-mediated arrest induction. The percentages of cells that shifted from G_1 to G_2 upon Vpr expression in *cdc2-1w* and *cdc2-3w* supported this conclusion and the hypothesis that both Wee1 and Cdc25 mediate Vpr-induced cell cycle arrest. In wild-type fission yeast, a dramatic (>80%) shift of G_1 cells to G_2 resulted by 40 hours post-induction of *vpr* expression; this shift was significantly smaller in *cdc2-1w* (~40%) and in *cdc2-3w* (~55%), indicating an

ability of both mutations to partially suppress the Vpr-associated arrest (92). Contrary to Masuda *et al.*'s findings, these *cdc2-3w* strain data suggested that Cdc25 might play a role in Vpr's effects on the fission yeast cell cycle.

In their second study, Elder *et al.* attempted to further delineate the requirements for *S. pombe* Wee1 and Cdc25 in Vpr-mediated G_2 arrest. The Vpr expression-associated G_1 to G_2 cell cycle shift in the wild-type strain was reduced by 66 +/- 15% in a delta*wee1* strain, supporting a major role for Wee1. This suppression was reported to be slightly higher in a *wee1-50* delta*cdc25* strain (83 +/- 12%) grown at the non-permissive temperature, a finding used to support the contention that Cdc25 contributes to Vpr's cell cycle effects in fission yeast. These data are inconclusive, however, as the statistical confidence intervals are widely overlapping. Data from an indirect *in vivo* enzymatic assay provided some evidence of a role for *S. pombe* Cdc25 as Vpr expression resulted in Cdc25 phosphatase inhibition. However, genetic data presented, but not discussed, in this second paper directly challenged Cdc25 involvement: Vpr was shown to be fully capable of inducing cell cycle arrest in their double mutant *cdc2-3w,* delta*cdc25* strain (93, Figure 3A). The authors concluded that Vpr both positively regulates Wee1 and negatively regulates Cdc25 to effect G_2/M arrest in fission yeast, with Wee1 activation playing the major role (93). The preponderance of evidence, however, suggests that Cdc25 is not involved in Vpr-mediated arrest initiation, but may be involved in its maintenance.

Elder *et al.* expressed Vpr in three additional *S. pombe* mutant strains: *pabl*, *dis2*, and *sds21*. Vpr-induced cell cycle arrest in the latter two, each lacking a different PP1 subunit, was reported to be indistinguishable from that in wild-type fission yeast,

suggesting that PP1, a family of okadaic acid-sensitive phosphatases, does not mediate Vpr-induced arrest. In *pab1*, however, deficient in the fission yeast PP2A regulatory B subunit, the Vpr-induced G_1 to G_2 shift was reduced to an even greater extent than that in *ppa2*, and the *cdc phenotype* suppression previously demonstrated in the *ppa2* strain was similarly observed in the Vpr-expressing *pab1* strain. This suggested involvement of not only the major *S. pombe* PP2A catalytic subunit, but also at least one of its regulatory subunits. Elder *et al.* further pursued the effects of Vpr expression on Ppa2, providing Western Blot data to suggest that Vpr up-regulates Ppa2 expression levels (93).

Other findings reported in the second Elder *et al.* study included identification of a multi-copy enhancer and a multi-copy suppressor of Vpr F34I-induced cell cycle arrest. These screens employed a fission yeast strain with a single-copy, chromosome-integrated, inducible gene encoding a Vpr with a single amino acid alteration, F34I. The mutated gene product retained wild-type Vpr cell cycle arrest function but was less toxic than the wild-type protein. This method allowed the investigators to limit Vpr expression levels and to focus more specifically on cellular mediators of the arrest function. Elder *et al.* noted that over-expression of Rad25, an *S. pombe* 14-3-3 protein homologue, enhanced Vpr F34I-induced G_2 arrest while over-expression of Wos2, the fission orthologue of the human p23 co-chaperone, suppressed Vpr F34I-induced G_2 arrest. The Rad25 finding was used as evidence of Cdc25 involvement as 14-3-3 proteins have been demonstrated to bind and inhibit Cdc25. This argument is not convincing, however, as the scope of 14-3-3 binding partners is broad and includes Wee1, which, as mentioned in the cell cycle regulation overview, is positively regulated by 14-3-3 binding.

In subsequent publications, this group and its collaborators provided data that also identified certain *S. pombe* heat shock proteins (Hsps), Hsp70 and two small Hsps, as multi-copy suppressors of Vpr F34I's ability to effect cell cycle arrest (93). Additionally, Zhao and Elder reported that over-expression of Sum1, originally observed to suppress the uncontrolled mitosis phenotype of the *cdc2-3w* strain, enhanced Vpr-induced arrest in *S. pombe*, but the source of the primary data demonstrating Sum1 enhancement is obscure (100).

Models of Vpr-induced Cell Cycle Arrest in S. pombe

Of the three Vpr-interacting genes identified by Masuda *et al.*, only the *wee1* product is a direct Cdc2 regulator; therefore, Wee1 was the central Vpr target in this group's model. Based on their *cdc phenotype* suppression data and findings of previous studies, Vpr-Wee1 direct interaction and Vpr-mediated Nim1 inhibition were ruled out as mechanisms causing the inferred Wee1 activation. This group instead speculated that Vpr might exert its effects on Wee1 by inhibiting a second Wee1 negative regulatory kinase, Cdr2, or by inhibiting Wee1 proteolysis and suggested that Vpr may target Wee1 through interactions with multiple pathways. Specifically, Vpr might positively influence Wee1 by modulating a PP2A regulatory pathway and a second, separate Rad24-dependent pathway. To support this interpretation of available data, their discussion emphasized previous findings demonstrating: (1) positive regulation of Wee1 by PP2A; (2) sensitivity of Vpr-mediated arrest in mammalian cells to okadaic acid, a potent PP2A inhibitor; and (3) a G_2/M cell cycle checkpoint pathway link between Rad24 and Wee1. Masuda *et al.* suggested that Vpr might exploit downstream components of this

checkpoint pathway to cause a Wee1-mediated G_2/M arrest without actually activating the checkpoint, and since PP2A had not been directly linked to this pathway, this group's model proposed Vpr-mediated Wee1 activation or stabilization through multiple mechanisms (91).

PP2A and its effects on multiple downstream targets were at the center of the Elder *et al.* model for Vpr-induced arrest. Because of the demonstrated role for *pab1* in this arrest and the broad range of B subunit family members in mammalian cells, potentially selective alterations in PP2A activities, based on B subunit substrate specificity, were suggested in this model. Vpr's effects on PP2A were speculated to occur through association with either (a) PP2A subunit(s) or a mediator of PP2A activation. The model also favored an important role for Vpr-induced PP2A catalytic subunit up-regulation in the cell cycle arrest-induction process.

Both Wee1 and Cdc25 were designated as important Vpr mediators: the model proposed that Vpr's positive influence on PP2A activity resulted in positive regulation of Wee1 and negative regulation of Cdc25. Elder *et al.* invoked the activity of PP2A-moderated dephosphorylation cascades upstream of both Cdc25 and Wee1 in linking Vpr with Wee1 and Cdc25. Kinoshita *et al.* had previously demonstrated that *S. pombe cdc25* and *wee1* interact genetically with *ppa2* (186). Like Cdk1, its regulators Wee1 and Cdc25 are regulated by activating and inactivating phosphorylation and dephosphorylation. While the pathways have not been totally defined, a substantial amount of evidence indicates that PP2A functions in pathways that stabilize Wee1 and inactivate Cdc25 from S phase until mitotic onset (186-191).

Elder *et al.* additionally included participation of Rad25, one role of which is to negatively regulate Cdc25, and Wos2, the over-expression of which inhibits Wee1. In a recent review; Zhao and Elder added Sum1 to their model as a proposed Cdc25 inhibitor and speculated that the PP2A-mediated mechanism by which Vpr arrests the cell cycle might also cause ATR activation in human cells (93, 100).

Discussion of Data and Models

Arguments that inherent differences between *S. pombe* and mammalian cell DNA damage responses and cell cycle progression regulation limit the ability to generalize fission yeast experimental findings to the HIV-1 natural infection situation are difficult to counter. However, the similarities between the experimental effects of *vpr* expression in mammalian and fission yeast cells suggest that their differences may not be particularly relevant. Since the human homologues of the identified *S. pombe* mediators have not been ruled out as participants in G_2 arrest of HIV-infected cells, the fission yeast findings warrant extensive consideration.

The data supporting roles for PP2A, Wee1, and 14-3-3 protein homologues in Vpr's arrest of the *S. pombe* and/or mammalian cell cycle are clear (91-93, 102, 105, 106). The genetic experiments of Masuda *et al.* and Elder *et al.* firmly establish roles for both the major PP2A catalytic and regulatory B subunits of fission yeast. While the role of PP2A has not been extensively examined in mammalian systems, early experiments demonstrating an okadaic acid-mediated reversal of Vpr's cell cycle effects suggest that the same may be true in human cell culture. In the experiments of Re *et al.*, okadaic acid treatment allowed rapid mitotic entry of both aphidicolin-treated and Vpr-expressing

HeLa cells. The authors implied that the mechanism by which this occurred involved dephosphorylation of inhibitory Cdk1 residues mediated by (an) okadaic acid-sensitive phosphatase (PP1 and/or PP2A) pathway(s). This, however, was not demonstrated (4). Data from subsequent Vpr cell cycle studies suggest that a more likely explanation: okadaic acid, specifically affecting PP2A function, prevented phosphorylation of Cdk1 residues by Wee1.

In addition to the fission yeast Wee1 evidence, Yuan *et al.* recently proposed that delayed Wee1 proteolysis may contribute to Vpr's arrest of the human cell cycle. Using a *Xenopus* egg extract system, Michael and Newport had previously demonstrated a requirement for Wee1 proteolysis for mitotic entry of cells with replicated genomes, and prevention of Wee1 degradation was identified as a mechanism for preventing mitotic entry prior to completion of essential S phase events (192). Yuan *et al.* provided evidence that Wee1 stabilization is essential for Vpr-mediated mammalian cell cycle arrest. Vpr expression doubled Wee1 protein half-life in G_1/S-synchronized HeLa cells while simultaneously reducing Cdk1 activity, and siRNA targeted to Wee1 mRNA blocked Vpr's ability to arrest cell cycle progression (66, 104).

In addition to the fission yeast evidence, data from mammalian cell experiments discussed in the next section strongly support a role for 14-3-3 proteins in Vpr's cell cycle effects (91, 93, 105, 106). Additionally, the recent finding in mammalian cells that PP2A and PP1 may function as important global regulators of 14-3-3 interactions (107) may lead to simplification of the Masuda *et al.* model. Instead of requiring separate Rad24- and PP2A-associated pathways for modulating Wee1 activity, their model may be explainable by Vpr's ability to disrupt a single pathway. Mediation of Vpr's effects by

human 14-3-3 proteins will be discussed in the next section along with the proposed roles of Cdc25 homologues. As mentioned above and contrary to the Zhao and Elder model, the preponderance of data currently suggests that Cdc25 is not an important component of Vpr-mediated arrest in fission yeast.

The over-expressed proteins identified as either Vpr F34I-induced arrest enhancers or suppressors in fission yeast are intriguing and may offer insight into mechanisms regulating this arrest in HIV-infected cells. One enhancer, Sum1, is essential for translation initiation and normal proliferation. This protein, highly similar to human transforming growth factor (TGF)-beta-receptor interacting protein 1 (TRIP1) and a eukaryotic translation initiation factor 3 (eIF3) subunit, interacts with the 40S ribosome, apparently functioning as a fission yeast eIF3 component. Experimental data suggest that Sum1 associates with the 26S proteasome in response to heat shock, indicating that Sum1 may functionally connect translation initiation with protein degradation and the cellular stress response (108).

In the context of Vpr-mediated cell cycle arrest, the most interesting Sum1 function may be its demonstrated interaction with the cellular stress response (108, 109). This response is an endogenous defense system triggered by exposure to any of a number of environmental stresses to promote cellular survival and to maintain or restore cellular homeostasis. Eukaryotic stress responses are generally products of: (1) rapid heat shock transcription factor (HSF) activation followed by HSF-mediated HSP expression induction and (2) mitogen-/stress-associated protein kinase (MAPK/SAPK) cascade activation. Each of the two stress response mechanisms regulate activities of the other, and both control cell cycle progression, especially the G_2/M transition, in response to

certain stresses [(110), reviewed in (111-115)]. Both Sum1 over-expression and SAPK pathway inactivation reverse the uncontrolled mitosis phenotype of *cdc2-3w* and inhibit osmotic stress-induced mitotic entry. Sum1 has also been demonstrated to interact with the fission yeast Sty1 SAPK pathway in regulating the G_2/M transitional response to environmental stress (109). These findings prompt speculation on possible Vpr interactions with eukaryotic SAPK pathways. The existence of such interactions might help unify the disparate models for Vpr-mediated cell cycle arrest, particularly in light of recent reports identifying SAPK activation-dependent G_2/M cell cycle checkpoints in both mammalian and fission yeast cells.

The identification of small Hsps as multi-copy suppressors of Vpr F34I-induced arrest also entices speculation on Vpr-mediated modulation of cellular stress responses. The major human small Hsp, HSP27, represents an important point of intersection for the two eukaryotic stress response mechanisms, *i.e.* HSF-mediated HSP expression induction and SAPK cascade activation. While HSP27 expression up-regulation requires HSF activation, functional activation of HSP27 requires MK2-catalyzed phosphorylation, and, therefore, p38 pathway activation (116, 117). That HSP27 dephosphorylation is effected primarily, if not solely, by PP2A is another finding potentially relevant to the mechanism(s) of Vpr-mediated activities (118) as is the demonstration that HSF1 is negatively regulated by 14-3-3 protein-mediated cytoplasmic sequestration (119).

Increasing evidence suggests that small Hsps modulate a number of Vpr activities in yeast and human cells. Gu *et al.* were first to examine interactions between Vpr and small Hsps (120). This group determined that dysregulation of the actin cytoskeleton was responsible for the Vpr expression-induced morphological defects in *Saccharomyces*

cerevisiae (*S. cerevisiae*) previously reported by Macreadie *et al.* (121) and demonstrated that over-expression of a budding yeast small Hsp, Hsp42p, reversed these defects (120). Recently, the Zhao laboratory and its collaborators demonstrated that over-expression of a fission yeast small Hsp, Hsp16, reversed multiple biological effects of Vpr. In addition to counteracting the VprF34I-induced *cdc phenotype* and proliferation defects, the gene encoding Hsp16 also functioned as a multi-copy suppressor of Vpr's cytotoxicity and of its ability to localize to the nuclear membrane. Subcellular co-localization and co-immunoprecipitation of epitope-tagged Vpr and Hsp16 from transformed fission yeast cells led the authors to prefer an interpretation that, when Hsp16 is present in sufficient quantities, Hsp16-Vpr physical interactions block Vpr activities, possibly by preventing Vpr interactions with other cellular proteins. The data did not, however, rule out the possibility that Hsp16 over-expression prevents an early post-infection, Vpr-dependent process required for multiple Vpr biological activities. Heterologous expression of *hsp16* resulted in suppression of Vpr activities in multiple human cell lines, including two $CD4^+$ lines in which HIV-1 replication was simultaneously suppressed (122). Bukrinsky and Zhao subsequently tested the hypothesis that HSP27 also inhibits Vpr biological activities and HIV-1 replication and provided preliminary data indicating that induction of HSP27 expression resulted in the restoration of a normal cell cycle distribution profile to HIV-1-infected cells (123).

wos2 (*wee1-50 over-expression suppressor 2*) was the first reported multi-copy suppressor of Vpr F34I-induced G_2 arrest in *S. pombe* (93). Munoz *et al.* had previously described Wos2 as being approximately 30% identical and orthologous to human p23, a co-chaperone component of HSP90 complexes. This group demonstrated that *wos2*

functionally interacts with both *wee1* and *cdc2* in mitotic entry regulation and provided data to suggest that Wos2 affects Cdc2's mitotic entry function in both Wee1-dependent and -independent fashions. Wos2 expression patterns suggested a proliferation-associated function: in cycling yeast cells, Wos2 protein levels were persistently high, but these dramatically decreased as cultures exited the growth phase. This function was not, however, essential under normal growth conditions as *wos2* deletion resulted in no significant negative effects on cellular viability and proliferation under such conditions, but *wos2* was characterized as a stress response gene as Wos2-deficient yeast were sensitive to hyperthermia (124, 125).

Mammalian co-chaperones like p23 are among the components of functional HSP90 complexes that have been implicated in regulating more than 100 client proteins. These include a number of essential transcription factors and signaling molecules, many of which may play roles in HIV-1 pathogenesis [(126), reviewed in (111-114)]. Swo1, the fission yeast Hsp90 chaperone, is essential for Wee1, Mik1, and Cdc2 function. Swo1 is required for Weel and Mik1 stabilization; in the absence of Swo1, Mik1 and Wee1 are hyper-susceptible to proteasome-mediated degradation. Swo1's effects on Cdc2 functions appear to be that of mediating Cdc2 protein-protein interactions necessary for mitotic progression (127). Munoz and Jimenez also demonstrated that *wos2* over-expression and expression of swo1-w1, a partial loss of function Swo1 mutant, increased Cdc2 activity synergistically, and these authors hypothesized that Wos2 physically associates with the Cdc2-Cdc13 complex to alter Cdc2 regulation by Hsp90 complexes (124, 125).

As with Sum1 homologues, no evidence has yet surfaced to suggest a role for p23 in Vpr-mediated arrest of HIV-1-infected cells. While Wos2 and the *S. cerevisiae* p23 orthologue, Sba1, are functionally interchangeable, human p23 may possess a different and/or broader scope of biological functions. In this regard, p23 was recently identified as the cytosolic prostaglandin E2 synthase (cPGES), an enzymatic function that is probably dependent on its association with HSP90 (128). If p23 is a mediator of Vpr's effects in human cells, alteration of a multitude of HSP90 complex regulatory functions might occur as the result of Vpr expression during HIV-1 infection, including altered prostaglandin production.

The remaining identified suppressor of Vpr F34I-mediated cell cycle arrest in *S. pombe*, Hsp70, is also a component of Hsp90 complexes (123). In response to cellular stress, human HSP70 isoforms promote cellular survival. Innate immune response-related roles for HSP70 have also been demonstrated (129-134). A number of experimental observations, including the significant HSP70 incorporation into HIV-1 virions, suggest that HSP70 may have important functions in HIV-1 virus-host interactions and in anti-viral responses (135-137). An interesting, evolving proposal is that Vpr shares functional similarities with HSP70 family members. However, the functional homology data supporting this proposal primarily center on the abilities of both Vpr and HSP70 to bind to importin-alpha and facilitate nuclear import, a Vpr function beyond the scope of this thesis (55, 138-140). Iordansky *et al.* even propose that HSP70 specifically targets Vpr to counteract this viral protein's functions, representing a unique type of anti-viral response. This group recently provided data in support of their hypothesis that HSP70 specifically prevents Vpr from effecting both cell cycle arrest and

apoptosis. While their proposition is thought-provoking, the cell cycle arrest suppression data supporting this hypothesis were not completely convincing (139, 140). Bukrinsky and Zhao's preliminary data suggested that over-expression of HSP27 was significantly more effective than that of an HSP70 isoform at reversing Vpr's cell cycle effects, and the data of the two subsequent Iordansky *et al.* studies similarly reflected incomplete reversal by HSP70 over-expression (123, 139, 140).

The fission yeast data generated important questions that remain unanswered. For example, what is the mechanism of Vpr-induced polyploidy, why does Vpr expression cause slow progression through S phase, and why is "abrogation" of Vpr-mediated G_2 arrest frequently not associated with restoration of cellular proliferation? The experimental observations giving rise to these questions support the Sherman *et al.* proposition that Vpr's cell cycle effects may not result from interactions with cell cycle regulators or pathways, but rather as an indirect result of its effects on (a) basic cellular process(es), *e.g.* DNA replication and repair, centrosome duplication, and membrane and cytoskeleton maintenance (141). These processes include, but are not limited to, As mentioned in the preceding section, Bartz *et al.* were first to demonstrate Vpr's ability to induce polyploidy in Jurkat T cells infected with *vpr*$^+$, but not *vpr*$^-$, experimental HIV strains. In these experiments, the Emerman group provided data demonstrating that a significant percentage of Vpr-expressing cells were capable of multiple rounds of DNA replication without a successful intervening cell division, and the presence of both mono- and multi-nucleated sub-populations of *8N vpr*$^+$ cells suggested that at least some cells had entered mitosis (90). Zhao *et al.* and Zhang *et al.* demonstrated a similar, but unexplained, occurrence in Vpr-expressing fission yeast: the DNA content in significant

percentages of these cells exceeded that expected of cells arrested at the G_2/M transition (102, 103). While the question of whether these cells had re-replicated DNA with or without entering mitosis was not pursued in these or any subsequent *S. pombe* studies, the characterization of Vpr's cell cycle effects as that of inducing G_2 or G_2/M arrest does not appear to adequately describe the spectrum of mitotic defects associated with its expression.

Recent data from Chang *et al.* emphasize the potential importance of the Bartz *et al.*'s findings to understanding the mechanism(s) of Vpr-mediated cell cycle arrest or delay. Chang *et al.* demonstrated that Vpr expression caused multiple defects in mitosis, nuclear division, and cytokinesis in fission yeast and in transformed (HeLa) and primary (endothelial) human cells. This group provided substantial evidence in support of their conclusion that Vpr's effects on cell division extend beyond its effects on cell cycle progression. Chang *et al.* also provided a convincing argument that Vpr's effects on cell cycle progression may be secondary to its effects on the mammalian centrosome and its yeast equivalent, the spindle pole body (SPB). Their results demonstrated that the Vpr-induced *cdc phenotype* was initiated at a later time than Vpr-induced mitotic spindle defects in *S. pombe*. In these experiments, Vpr disrupted not only centrosome/SPB organization and function, but also the centrosome/SPB duplication, separation, and segregation cycle (142). This cycle is stringently coordinated with that of cell division, and essential cell cycle regulators, including the Cdk1/cyclin B complex, are intimately associated with the SPB/centrosome [reviewed in (143-145)]. In at least some cell types, the centrosome may even function as the location for Cdk1/cyclin B final activation (146). Plo1, the fission yeast homologue of a previously-mentioned ATR target, Plk1,

was one aberrantly localized SPB component in Vpr-expressing *S. pombe*. Plo1 homologues demonstrate a broad range of conserved functions in cell division [reviewed in (147-149)], and, since *S. pombe* dysfunctional plo1 mutants exhibit defects similar to those resulting from Vpr expression (150), Chang *et al.* proposed that some or all of Vpr's effects may result from altered Plo1 regulation (142).

Zhao *et al.* and Zhang *et al.* disagreed on the cell cycle stage(s) at which Vpr arrested the fission yeast cell cycle, but, as mentioned earlier, the latter study's explanation for arrest at multiple stages was definitely plausible (102, 103). This explanation did not, however, explain their observation that progression of Vpr-expressing cells through S phase was significantly slower than that of control cells (103), and unexplained data from Yuan *et al.* seem to suggest that Vpr may similarly affect S phase progression in human cells. In their experiments, Wee1 mRNA levels in control virus-infected HeLa cells peaked at 4 hours post-release from G1/S synchronization while this peak did not occur until 2 hours later in vpr^+ virus-infected cells (104, Figure 3). In the absence of evidence to suggest that Vpr specifically affects Wee1 mRNA production, the most plausible interpretation of the data is that cellular processes during S phase were delayed by Vpr expression (104).

Also calling the specificity of Vpr's G_2/M effects into question was Masuda *et al.*'s observation that restoration of proliferation potential did not accompany reversion of the Vpr-induced *cdc phenotype* in the *S. pombe* mutant strains (91). In the absence of data to suggest a differential Vpr effect on cell viability, these data imply that Vpr-induced arrest or delay occurred at (a) stage(s) other than G_2/M in these strains. The possibility that Vpr affects progression to and through multiple cell cycle stages not only

suggests that these effects may be secondary to effects on another process, but also complicates interpretation of experimental data from many Vpr cell cycle studies. Most flow cytometry analyses from Vpr studies have typically ignored S phase, aneuploid, and polyploid cell populations by reporting Vpr-induced alterations in terms of G_1 to G_2 percentage shifts or G_2 to G_1 ratios.

Cdc25/Cdc25C Inhibition-dependent Models for Vpr-mediated G_2/M Arrest

Summary of Relevant Data

Re *et al.* were first to report that, in addition to Cdk1, Cdc25C was also inactive in Vpr-expressing mammalian cells (4). Previous work of Levy *et al.* and Rogel *et al.* had demonstrated that Vpr expression, while advantageous for viral propagation *in vivo*, prevented cellular replication in cell culture (1, 2), and Re *et al.* established that *vpr* expression led to HeLa cell cycle arrest in G_2. Their findings included: (1) a majority of their *vpr*-expressing HeLa cells possessed replicated genomes; (2) the Cdk1-cyclin B1 complexes immunoprecipitated from these cells were unable to phosphorylate histone H1 and contained inactive, Tyr^{15}-phosphorylated Cdk1; and (3) Cdc25C hypo-phosphorylation status was consistent between Vpr-expressing cells and the S-phase-arrested, aphidicolin-treated cells and not with those that had undergone mitotic entry post-nocodazole treatment.

Recently, Goh *et al.* reproduced and expanded upon these Cdc25 findings. In their experiments, active Cdc25C was not detectable in vpr^+-HIV-1-infected T cells despite the absence of observable change in total Cdc25C levels. Goh *et al.* pursued identification of the mechanism underlying the Cdc25C phosphatase inhibition and

provided data that suggested a physical interaction between Vpr and Cdc25C both *in vitro* and in mammalian cell cultures. GST-fused Cdc25C was able to efficiently bind *in vitro*-translated wild-type Vpr, and the degree of binding of this fusion protein to single-amino acid Vpr mutants correlated with the ability of each mutant to arrest the cell cycle. In transfected 293T cells, epitope-tagged Vpr was also able to co-immunoprecipitate some quantity of Cdc25C, and Cdc25C mutation and deletion analysis mapped the GST-Cdc25C's Vpr-binding domain to a 46-amino acid region in Cdc25C's carboxyl terminus (C-terminus) located upstream of its catalytic domain (99).

Vpr-mediated *in vitro* suppression of Cdc25C catalytic activity was also demonstrated in this study. Recombinant, histidine-tagged Vpr inhibited the activity of another GST fusion protein that contained an NH_2-terminally truncated Cdc25C, towards 3-*O*-methylfluorescein phosphate (OMFP), a chemical substrate of Cdc25C. A similarly-tagged Vpr mutant with defective G_2 arrest-inducing function and impaired Cdc25C-binding ability did not inhibit this ability, suggesting that wild-type Vpr's effects in this assay were due to a physical association with and inhibition of the Cdc25C's enzymatic activity localized to the Cdc25C C-terminus. Similar results were obtained in an *in vitro* assay of the ability of the truncated Cdc25C fusion to activate CDK1-cyclin B towards one of its physiological substrates, the histone H1 protein, in the presence of histidine-tagged wild-type versus mutant Vpr (99).

To demonstrate the necessity of Cdc25C in Vpr-mediated G_2 arrest, Goh *et al.* performed a number of manipulations in Vpr-expressing HeLa cells, each of which was predicted to reverse cell cycle arrest to varying degrees. These manipulations included over-expression of wild-type Cdc25C; over-expression of an active Cdc25C mutant with

impaired Vpr-binding ability; over-expression of an inactive, Vpr-binding Cdc25C mutant; and small, interfering RNA-mediated depletion of endogenous Cdc25C. Flow cytometric data indicated that each of these manipulations decreased the G_2/G_1 ratio of its cell population (99).

Other recent data have also implicated Cdc25C as a target of Vpr, but not a direct one. Instead, these data suggest that Cdc25C inhibition occurred secondary to a Vpr-14-3-3 protein association. As mentioned in the previous section, fission yeast genetics studies of Masuda *et al.* and Elder *et al.* provided the first evidence of a possible role for 14-3-3 proteins in mediating Vpr's cell cycle effects (91, 93). Very recently, Kino *et al.* have suggested the direct binding of Vpr with human 14-3-3 isoforms. This group performed a series of yeast two-hybrid screens, using wild-type and two mutant Vpr "baits" and HeLa cell cDNA libraries, to identify Vpr-interacting HeLa cell gene products that specifically lost the ability to interact with Vpr R80A, a cell cycle arrest-incompetent Vpr mutant. 14-3-3eta and 14-3-3sigma represented 7 of ninety-six total clones sequenced from the screen, and peptide fragment and mutational analysis of these isoforms suggested that their C-terminal regions interact with Vpr. Successful GST-14-3-3eta pull-down of wild-type Vpr and cell cycle arrest-competent Vpr mutants was demonstrated. 14-3-3eta appeared to enhance Vpr-mediated G_2 arrest in transient co-transfection experiments as evidenced by higher G_2/G_1 ratios in HeLa and 293 cells expressing both proteins than in those expressing Vpr alone. Additionally, the group provided co-immunoprecipitation data to suggest that transfected Vpr forms ternary complexes with endogenous Cdc25C and 14-3-3 proteins in 293 cells (105).

Kino *et al.*'s most interesting results may have been those obtained in wild-type and 14-3-3sigma$^{-/-}$ HCT116 cells. Infection of these cell lines with *vpr*$^{+}$ or *vpr*$^{-}$ HIV-1 molecular clones demonstrated that Vpr expression in the wild-type HCT116 resulted in a substantially larger G_2/G_1 cell population ratio than did its expression in HCT116 14-3-3sigma$^{-/-}$ cells. Knockout of 14-3-3sigma reduced the G_2/G_1 ratio from approximately 8 to 3 times that of controls, a result the authors assessed to be a nearly complete reversal of Vpr's mitotic entry block; and reestablishment of 14-3-3sigma expression in the knockout cells fully restored Vpr's capacity to induce G_2 arrest. Kino *et al.* tested Vpr's effects on CDC25C localization by immunofluorescence of wild-type and 14-3-3sigma$^{-/-}$ HCT116 cells expressing either enhanced green fluorescent protein (EGFP)-fused wild-type or Ser^{216}-mutant Cdc25C in the presence or absence of Vpr. Comparisons of the nuclear versus cytoplasmic EGFP signal ratios of these cell populations indicated the possible involvement of 14-3-3sigma in Vpr-mediated, Ser^{216} phosphorylation-independent, cytoplasmic retention of Cdc25C (105).

Discussion of Vpr-CDC25C Direct Interaction Model of Cell Cycle Arrest

Goh *et al.* suggest that direct association of Vpr with Cdc25C leads to G_2 arrest, a mechanism that might appear to be the simplest yet proposed for Vpr-mediated cell cycle arrest. However, in an exceptionally well-balanced discussion of their findings, Goh *et al.* point out the uncertainties created by these results in the context of others' work. Their recognition of the paradox generated by their data and that of Chen *et al.* was particularly insightful. Chen *et al.* had demonstrated that somatic cells from *cdc25C* knockout mice entered mitosis normally in response to proliferative signals while

retaining normal DNA checkpoint responses (151), but Goh *et al.*'s data indicated that CDC25C was essential for Vpr's prevention of mitotic entry (99). Since Chen *et al.* had suggested that either of two other mammalian Cdc25 homologues, Cdc25A or Cdc25B, was responsible for activating Cdk1 in the absence of Cdc25C (151), Goh *et al.* speculated that not only is CDC25C in complex with Vpr incapable of activating CDK1 but also prevents at least one other CDC25 homologue from doing the same, possibly via binding of the CDC25C-Vpr complex to CDK1-cyclin B (99).

Goh *et al.* ultimately concluded that their data support a role for aberrant regulation of CDC25C activity in Vpr-mediated arrest, but avoided overstating the significance of their results. This group stressed that few, if any, mechanistic details of Vpr-mediated arrest are known and specifically cited the previously discussed fission yeast and Re *et al.* as particularly important to elucidating these details. In doing so, Goh *et al.* stated or implied that any proposed detailed mechanism must account for PP2A, Wee1, and 14-3-3 protein involvement in addition to that of CDC25C. The Vpr-mediated disruption of normal spatial regulation of cell cycle regulatory molecules suggested by de Noronha *et al.*, particularly their nuclear and cytoplasmic compartmentalization and trafficking, was another level of potentially significant dysregulation discussed by these authors (4, 91-93, 98, 99).

While Goh *et al.*'s discussion placed the significance of its findings within an appropriate context, the limitations of their published data require discussion. The primary criticism is not unique to this study and involves the method of analyzing Vpr expression-related flow cytometric data. Comparisons of G_2/G_1 ratios at single time points post-experimental manipulation is inadequate for gaining a clear understanding of

the Vpr's cell cycle effects. In two studies, the Aida group demonstrated that Vpr was capable of preventing cellular proliferation by a mechanism independent of G_2/M arrest induction, possibly involving G_1 arrest induction (152, 153). This, along with evidence of Vpr's propensity for inducing both S-phase progression delays and polyploidy, complicates interpretation of single time-point, G_2/G_1 ratio data as does the possible occurrence of differential, experimental manipulation-dependent cytotoxic effects.

Another criticism applicable, but not restricted, to the Goh *et al.* study is that of assessing the biological relevance of co-immunoprecipitation demonstrations involving Vpr. Dozens of Vpr-cellular protein associations linked with Vpr's ability to influence cell cycle progression have been identified by this method. Each of these demonstrated interactions has been accompanied by genetic and/or biochemical evidence to support biological relevance. While highly improbable that all of these interactions are truly relevant in the context of natural HIV-1 infection, ascertaining which one(s) is/are truly important is impossible. Insofar as the Goh *et al.* data, however, any interpretation that suggests Vpr's cell cycle effects are due solely to its interactions with the Cdc25 phosphatases is, at the very least, premature and is probably incorrect.

14-3-3-dependent CDC25C Inhibition Model of Vpr-mediated Arrest

Kino *et al.* concluded that their data supported a model whereby Vpr affects cell cycle arrest by binding to 14-3-3 proteins. In this model, Vpr-14-3-3 interactions enhance the ability of 14-3-3 proteins to inhibit Cdc25C by allowing 14-3-3-Cdc25C association without prerequisite Cdc25C Ser^{216} phosphorylation. These Vpr-14-3-3

associations with Cdc25C are proposed to result in Cdc25C cytoplasmic sequestration and thereby prevent Cdc25C's CDK1-cyclin B complex-activating ability (105).

Kino *et al.*'s data implicating 14-3-3 protein involvement in Vpr-mediated arrest was compelling, especially considering the *S. pombe* data suggesting similar involvement. However, the data did not support the proposed model, particularly the data of the HCT116 cell experiments. Wild-type Cdc25C localization did not appear to be affected by Vpr and/or 14-3-3sigma expression in apparent contradiction of the cell cycle arrest data. That cell cycle arrest data, assuming the model is correct, should have led to at least two predictions not supported by the immunolocalization studies. First, since Vpr expression in wild-type HCT116 cells resulted in a dramatically increased G_2/G_1 ratio indicative of a G_2 arrest, Vpr expression in these wild-type Cdc25C-expressing cells should have resulted in an EGFP-Cdc25C cytoplasmic shift. Second, since Vpr expression in the absence of 14-3-3sigma in the knockout cells was still associated with a three-fold higher G_2/G_1 ratio than both Vpr^- control populations (wild-type and 14-3-3sigma knockout HCT116 cells), the cytoplasmic/nuclear EGFP-Cdc25C ratios should have reflected these differences as well. Because the first of these predictions, in particular, was not satisfied by the data, the Cdc25C Ser^{216} mutant relocalization data, while interesting, is moot. While the Vpr-14-3-3 protein direct interactions appear to be biologically relevant, Kino *et al.*'s model is flawed.

How biologically relevant is the formation of Vpr-14-3-3 protein-Cdc25C ternary complexes and Cdc25C inhibition? The most interesting Kino *et al.* finding, the dramatic effect of 14-3-3sigma knockout on Vpr's ability to arrest human colon carcinoma-derived

HCT116 cells (105), opposes the interpretation of a prominent role for Cdc25C inhibition in Vpr-mediated arrest. HCT116 14-3-3sigma promotes DNA damage-induced G_2 arrest and prevents DNA damage-induced mitotic catastrophe in a Cdc25C-independent manner (19, 154, 155). The method by which 14-3-3sigma performs these functions has not been clearly defined despite initial suggestions that these effects were the product of direct 14-3-3sigma interaction with Cdk1-cyclin B complexes (19). While such a mechanism is would be difficult to reconcile with Vpr-mediated effects, Kino *et al.*'s data demonstrating that 14-3-3sigma loss prevents Vpr-mediated arrest without changing wild-type Cdc25C distribution patterns suggest that 14-3-3sigma may modulate cellular responses to Vpr expression and DNA damage by a similar mechanism. Excitement over the 14-3-3sigma$^{-/-}$ Vpr data may be somewhat dampened by the demonstration that the 14-3-3sigma isoform appears to be primarily important in epithelial cells (156, 157), the possibility cannot be excluded that Vpr affects a similar mechanism in immune system cells, possibly involving (a) different 14-3-3 isoform(s).

CONCLUSION

The concluding statement of Goh *et al.*'s discussion on the role of Cdc25 homologues in Vpr-mediated arrest is undeniably true. The mechanism of Vpr-mediated arrest will remain elusive until we acquire a more detailed understanding of the interconnections among the cellular pathways and processes that influence key cell cycle regulatory molecules (99). Currently available data suggest important roles for PP2A, 14-3-3 proteins, Wee1, and Cdc25 homologues in this process (4, 91, 93, 100, 158), but the detailed molecular mechanism of those roles is unknown . Direct interactions with

Vpr have been reported for three of the four, excepting Wee1 (99, 105, 158); the one report of a PP2A subunit-Vpr interaction was, however, ultimately retracted (158). The data suggesting involvement of a direct Vpr-Cdc25C interaction are not easily incorporated into a single arrest mechanism. Since 14-3-3 proteins and PP2A interact, reviewed in (159), and both regulate Cdc25 homologues and Wee1, (160-164) and reviewed in (81), a direct interaction between either and Vpr offers a reasonable, relatively simple explanation for Vpr-mediated arrest. Demonstration of a biologically relevant direct interaction between Vpr and either or both fission yeast 14-3-3 homologues would contribute greatly to integrating the wealth of data available in the field of Vpr-mediated cell cycle arrest.

While Vpr does induce G_2/M arrest in a majority of cells, this outcome of Vpr expression appears to be a product of alterations that begin in early S phase (66, 91, 103, 104, 152, 153). G_2/M arrest induction may not be a specific intent of HIV-1 Vpr, but rather the typical cellular response to disruption of cellular processes by this protein. Vpr-associated experimental findings such as re-duplication of the genome in the absence of nuclear division and induction centrosome duplication defects prior to arrest induction support such a view (90, 142). The S phase timing of alteration onset and subsequent activation of ATR suggest that Vpr may target early steps in the DNA replication process, and "DNA licensing" factor involvement is suggested by the genome re-duplication findings. *S. pombe* data demonstrating that suppression of Vpr-mediated arrest in *ppa2*, *rad24*, and *wee1* mutant strains did not result in restoration of cell proliferation (91) indicate that each of these gene products either participate in or are influenced by the cellular processes affected by Vpr expression. Investigation of

potential 14-3-3 and PP2A roles in DNA replication initiation and DNA licensing may offer particularly enlightening results.

The source of Vpr's cell cycle effects appears to affect a number of additional cellular processes and pathways. How the regulation of these with one another and with those mentioned above offers a vast field for exploration. Prompted primarily by the de Noronha *et al.* demonstration of Vpr-generated nuclear envelope abnormalities (98), Sherman *et al.* had first suggested that Vpr's cell cycle effects might be secondary to effects on another cellular process rather than a product of a direct association with a cell cycle regulatory molecule (141). In addition to disturbed membrane function, Vpr may affect a number of cellular processes with regulatory ties to the cell cycle. These include centrosome duplication cycle dysregulation, and plo1/plk1 might provide a link between the two (97, 142, 165-167). Vpr may also affect the translation initiation process as two eukaryotic translation initiation factor 3 (eIF3) components have now been implicated in Vpr-mediated arrest, human Mov34 and fission yeast Sum1 (100, 168, 169). Sum1 and Vpr have also been implicated in proteasomal degradation processes (108, 170, 171), and proteasomal degradation is a major mechanism for controlling the activity of cell cycle regulators. DNA repair processes may be associated with Vpr's cell cycle effects. In addition to Zimmerman *et al.*'s demonstration of Vpr-associated BRCA1 and H2AX redistribution (97), direct interactions with two excision repair proteins, UNG and HHR23A, have been demonstrated (51, 172-178); and the interaction with HHR23A has been associated with Vpr's ability to arrest the cell cycle (176). Regulation of cytoskeleton function and MAPK signaling (68, 120, 121, 164, 179) are also among the processes affected by Vpr with links to cell cycle regulation. While the mechanism(s) of

Vpr-mediated arrest are incompletely understood, the number of processes linked to Vpr-mediated arrest seems to indicate that Vpr directly interacts with a cellular component involved in regulating a vast array of processes to exert its effects. 14-3-3 proteins and PP2A are such cellular components.

Vpr's roles in modifying intracellular conditions to the advantage of the virus, particularly in coordinating viral life cycle progression with cell cycle progression, that . Env induces activation of a number of signaling pathways by ligating CD4 and the chemokine co-receptor, and the infected cell displays a propensity to undergo apoptosis as the result of Env-mediated events, reviewed in (180). The concept that Vpr, as a viral immediate early protein (181), might be important in promoting infected cell survival by dampening these effects is an attractive one. Further investigations into how early events of the viral life, *e.g.* reverse transcription, movement of the PIC to and through the nuclear envelope, and proviral integration, are coordinated with the host cell cycle progression may give a clearer picture of Vpr's value to viral propagation.

REFERENCES

1. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. J. Virol. **69**:882-888.
2. **Levy, D. N., L. S. Fernandes, W. V. Williams, and D. B. Weiner.** 1993. Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell **72**:541-550.
3. **He, J., S. Choe, R. Walker, P. Di Marzio P, D. O. Morgan, and N. R. Landau.** 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. **69**:6705-6711.
4. **Re, F., D. Braaten, E. K. Franke, and J. Luban.** 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. J. Virol. **69**:6859-6864.
5. **Jowett, J. B., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Chen.** 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J. Virol. **69**:6304-6313.
6. **Dedera, D., W. Hu, N. Vander Heyden, and L. Ratner.** 1989. Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. J. Virol. **63**:3205-3208.
7. **Tristem, M., C. Marshall, A. Karpas, and F. Hill.** 1992. Evolution of the primate lentiviruses: evidence from vpx and vpr. EMBO J. **11**:3405-3412.
8. **Goh, W. C., M. E. Rogel, C. M. Kinsey, S. F. Michael, P. N. Fultz, M. A.**

Nowak, B. H. Hahn, and M. Emerman. 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat. Med. **4**:65-71.

9. **Forget, J., X. J. Yao, J. Mercier J, and E. A. Cohen.** 1998. Human immunodeficiency virus type 1 vpr protein transactivation function: mechanism and identification of domains involved. J. Mol. Biol. **284**:915-923.

10. **Subbramanian, R. A., A. Kessous-Elbaz, R. Lodge, J. Forget, X. J. Yao, D. Bergeron, and E. A. Cohen.** 1998. Human immunodeficiency virus type 1 Vpr is a positive regulator of viral transcription and infectivity in primary human macrophages. J. Exp. Med. **187**:1103-1111.

11. **Felzien, L. K., C. Woffendin, M. O. Hottiger, R. A. Subbramanian, E. A. Cohen, and G. J. Nabel.** 1998. HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 co-activator. Proc. Natl. Acad. Sci. USA **95**:5281-5286.

12. **Gummuluru, S. and M. Emerman.** 1999. Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. J. Virol. **73**:5422-5430.

13. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. J. Acquir. Immune Defic. Syndr. **3**:11-18.

14. **Agostini, I., J. M. Navarro, F. Rey, M. Bouhamdan, B. Spire, R. Vigne, and J. Sire.** 1996. The human immunodeficiency virus type 1 Vpr transactivator: cooperation with promoter-bound activator domains and binding to TFIIB. J. Mol.

Biol. **261**:599-606.

15. **Conti, L., P. Matarrese, B. Varano, M. C. Gauzzi, A. Sato, W. Malorni, F. Belardelli, and S. Gessani.** 2000. Dual role of the HIV-1 vpr protein in the modulation of the apoptotic response of T cells. J. Immunol. **165**:3293-3300.

16. **Ayscough, K., J. Hayles, S. A. MacNeill, and P. Nurse.** 1992. Cold-sensitive mutants of p34cdc2 that suppress a mitotic catastrophe phenotype in fission yeast. Mol. Gen. Genet. **232**:344-350.

17. **Campbell, S. D., F. Sprenger, B. A. Edgar, and P. H. O'Farrell.** 1995. Drosophila Wee1 kinase rescues fission yeast from mitotic catastrophe and phosphorylates Drosophila Cdc2 in vitro. Mol. Biol. Cell **6**:1333-1347.

18. **Bueno, A. and P. Russell.** 1993. Two fission yeast B-type cyclins, cig2 and Cdc13, have different functions in mitosis. Mol. Cell Biol. **13**:2286-2297.

19. **Chan, T. A., H. Hermeking, C. Lengauer, K. W. Kinzler, and B. Vogelstein.** 1999. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature **401**:616-620.

20. **Molz, L., R. Booher, P. Young, and D. Beach.** 1989. cdc2 and the regulation of mitosis: six interacting mcs genes. Genetics **122**:773-782.

21. **Fukumori, T., H. Akari, S. Iida, S. Hata, S. Kagawa, Y. Aida, A. H. Koyama, and A. Adachi.** 1998. The HIV-1 Vpr displays strong anti-apoptotic activity. FEBS Lett. **432**:17-20.

22. **Conti, L., G. Rainaldi, P. Matarrese, B. Varano, R. Rivabene, S. Columba, A. Sato, F. Belardelli, W. Malorni and S. Gessani.** 1998. The HIV-1 vpr protein acts as a negative regulator of apoptosis in a human lymphoblastoid T cell line:

possible implications for the pathogenesis of AIDS. J. Exp. Med. **187**:403-413.

23. **Ayyavoo, V., A. Mahboubi, S. Mahalingam, R. Ramalingam, S. Kudchodkar, W. V. Williams, D. R. Green, and D. B. Weiner.** 1997. HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. Nat. Med. **3**:1117-1123.

24. **Le Rouzic, E. and S. Benichou**. 2005. The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology **2**:11.

25. **Lehmann-Che, J. and A. Saib.** 2004. Early stages of HIV replication: how to hijack cellular functions for a successful infection. AIDS Rev. **6**:199-207.

26. **Moser, B. A. and P. Russell.** 2000. Cell cycle regulation in Schizosaccharomyces pombe. Curr. Opin. Microbiol. **3**:631-636.

27. **Smits, V. A. and R. H. Medema.** 2001. Checking out the G(2)/M transition. Biochim. Biophys. Acta **1519**:1-12.

28. **Pagano, M. and P. K. Jackson.** 2004. Wagging the dogma; tissue-specific cell cycle control in the mouse embryo. Cell **118**:535-538.

29. **Obaya, A. J. and J. M. Sedivy.** 2002. Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol. Life Sci. **59**:126-142.

30. **Wang, W. K., M. Y. Chen, C. Y. Chuang, K. T. Jeang, and L. M. Huang.** 2000. Molecular biology of human immunodeficiency virus type 1. J. Microbiol. Immunol. Infect. **33**:131-140.

31. **Pines, J.** 1999. Four-dimensional control of the cell cycle. Nat. Cell Biol. **1**:E73-79.

32. **Arrigo, S. J. and I. S. Chen.** 1991. Rev is necessary for translation but not

cytoplasmic accumulation of HIV-1 vif, vpr, and env/vpu 2 RNAs. Genes Dev. **5**:808-819.

33. **Furtado, M. R., R. Balachandran, P. Gupta, and S. M. Wolinsky.** 1991. Analysis of alternatively spliced human immunodeficiency virus type-1 mRNA species, one of which encodes a novel tat-env fusion protein. Virology **185**:258-270.

34. **Gras-Masse, H., J. C. Ameisen, C. Boutillon, J. C. Gesquiere, S. Vian, J. L. Neyrinck, H. Drobecq, A. Capron, and A. Tartar**. 1990. A synthetic protein corresponding to the entire vpr gene product from the human immunodeficiency virus HIV-1 is recognized by antibodies from HIV-infected patients. Int. J. Pept. Protein Res. **36**:219-226.

35. **Engler, A., T. Stangler, and D. Willbold.** 2002. Structure of human immunodeficiency virus type 1 Vpr(34-51) peptide in micelle containing aqueous solution. Eur. J. Biochem. **269**:3264-3269.

36. **Cornille, F., K. Wecker, A. Loffet, R. Genet, and B. Roques.** 1999. Efficient solid-phase synthesis of Vpr from HIV-1 using low quantities of uniformly 13C-, 15N-labeled amino acids for NMR structural studies. J. Pept. Res. **54**:427-435.

37. **Engler, A., T. Stangler, and D. Willbold.** 2001. Solution structure of human immunodeficiency virus type 1 Vpr(13-33) peptide in micelles. Eur. J. Biochem. **268**:389-395.

38. **Morellet, N., S. Bouaziz, P. Petitjean, and B. P. Roques.** 2003. NMR structure of the HIV-1 regulatory protein VPR. J. Mol. Biol. **327**:215-227.

39. **Schuler, W., K. Wecker, H. de Rocquigny, Y. Baudat Y, J. Sire, and B. P.**

Roques. 1999. NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: molecular insights into its biological functions. J. Mol. Biol. **285**:2105-2117.

40. **Roques, B. P., N. Morellet, H. de Rocquigny, H. Demene, W. Schueler, and N. Jullian.** 1997. Structure, biological functions and inhibition of the HIV-1 proteins Vpr and NCp7. Biochimie **79**:673-680.

41. **Wecker, K. and B. P. Roques.** 1999. NMR structure of the (1-51) N-terminal domain of the HIV-1 regulatory protein Vpr. Eur. J. Biochem. **266**:359-369.

42. **Wecker, K., N. Morellet, S. Bouaziz, and B. P. Roques.** 2002. NMR structure of the HIV-1 regulatory protein Vpr in H2O/trifluoroethanol. Comparison with the Vpr N-terminal (1-51) and C-terminal (52-96) domains. Eur. J. Biochem. **269**:3779-3788.

43. **Sherman, M. P., U. Schubert, S. A. Williams, C. M. de Noronha, J. F. Kreisberg, P. Henklein, and W. C. Greene.** 2002. HIV-1 Vpr displays natural protein-transducing properties: implications for viral pathogenesis. Virology **302**:95-105.

44. **Henklein, P., K. Bruns, M. P. Sherman, U. Tessmer, K. Licha, J. Kopp, C. M. de Noronha, W. C. Greene, V. Wray, and U. Schubert.** 2000. Functional and structural characterization of synthetic HIV-1 Vpr that transduces cells, localizes to the nucleus, and induces G2 cell cycle arrest. J. Biol. Chem. **275**:32016-32026.

45. **Huang, M. B., O. Weeks, L. J. Zhao, M. Saltarelli, and V. C. Bond.** 2000. Effects of extracellular human immunodeficiency virus type 1 vpr protein in primary rat cortical cell cultures. J. Neurovirol. **6**:202-220.

46. **Azad, A. A.** 2000. Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? Biochem. Biophys. Res. Commun. **267**:677-685.

47. **Macreadie, I. G., C. K. Arunagiri, D. R. Hewish, J. F. White, and A. A. Azad.** 1996. Extracellular addition of a domain of HIV-1 Vpr containing the amino acid sequence motif H(S/F)RIG causes cell membrane permeabilization and death. Mol. Microbiol. **19**:1185-1192.

48. **Kino, T. and G. N. Pavlakis.** 2004. Partner molecules of accessory protein Vpr of the human immunodeficiency virus type 1. DNA Cell Biol. **23**:193-205.

49. **Tungaturthi, P. K., B. E. Sawaya, V. Ayyavoo, R. Murali, and A. Srinivasan.** 2004. HIV-1 Vpr: genetic diversity and functional features from the perspective of structure. DNA Cell Biol. **23**:207-222.

50. **Stark, L. A. and R. T. Hay.** 1998. Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) interacts with Lys-tRNA synthetase: implications for priming of HIV-1 reverse transcription. J. Virol. **72**:3037-3044.

51. **Mansky, L. M., S. Preveral, L. Selig, R. Benarous, and S. Benichou.** 2000. The interaction of vpr with uracil DNA glycosylase modulates the human immunodeficiency virus type 1 In vivo mutation rate. J. Virol. **74**:7039-7047.

52. **Mansky, L. M.** 1996. The mutation rate of human immunodeficiency virus type 1 is influenced by the vpr gene. Virology **222**:391-400.

53. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1

influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91**:7311-7315.

54. **Popov, S., M. Rexach, G. Zybarth, N. Reiling, M. A. Lee, L. Ratner, C. M. Lane, M. S. Moore, G. Blobel, and M. Bukrinsky.** 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J. **17**:909-917.

55. **Vodicka, M. A., D. M. Koepp, P. A. Silver, and M. Emerman.** 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. **12**:175-185.

56. **Wang, L., S. Mukherjee, F. Jia, O. Narayan, and L. J. Zhao.** 1995. Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and trans-activation of viral long terminal repeat. J. Biol. Chem. **270**:25564-25569.

57. **Refaeli, Y., D.N. Levy, and D. B. Weiner.** 1995. The glucocorticoid receptor type II complex is a target of the HIV-1 vpr gene product. Proc. Natl. Acad. Sci. USA **92**:3621-3625.

58. **Sawaya, B. E., K. Khalili, J. Gordon, R. Taube, and S. Amini.** 2000. Cooperative interaction between HIV-1 regulatory proteins Tat and Vpr modulates transcription of the viral genome. J. Biol. Chem. **275**:35209-35214.

59. **Muthumani, K., A. Y. Choo, D. S. Hwang, M. A. Chattergoon, N. N. Dayes, D. Zhang, M. D. Lee, U. Duvvuri, and D. B. Weiner.** 2003. Mechanism of HIV-1 viral protein R-induced apoptosis. Biochem. Biophys. Res. Commun. **304**:583-592.

60. **Stewart, S.A., B. Poon, J. B. Jowett, and I. S. Chen.** 1997. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. J. Virol. **71**:5579-5592.

61. **Stewart, S.A., B. Poon, J. Y. Song, and I. S. Chen.** 2000. Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. J. Virol. **74**:3105-3111.

62. **Vieira, H. L., D. Haouzi, C. El Hamel, E. Jacotot, A. S. Belzacq, C. Brenner, and G. Kroemer.** 2000. Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ. **7**:1146-1154.

63. **Roumier, T., H. L. Vieira, M. Castedo, K. F. Ferri, P. Boya, K. Andreau, S. Druillennec, N. Joza, J. M. Penninger, B. Roques, and G. Kroemer**. 2002. The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway. Cell Death Differ. **9**:1212-1219.

64. **Brenner, C. and G. Kroemer.** 2003. The mitochondriotoxic domain of Vpr determines HIV-1 virulence. J. Clin. Invest. **111**:1455-1457.

65. **Jacotot, E., L. Ravagnan, M. Loeffler, K. F. Ferri, H. L. Vieira, N. Zamzami, P. Constantini, S. Druillennec, J. Hoebeke, J. P. Briand, T. Irinopoulou, E. Daugas, S. A. Susin, D. Cointe, Z. H. Xie, J. C. Reed, B. P. Roques, and G. Kroemer.** 2000. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J. Exp. Med. **191**:33-46.

66. **Yuan, H., Y. M. Xie, and I. S. Chen.** 2003. Depletion of Wee-1 kinase is

necessary for both human immunodeficiency virus type 1 Vpr- and gamma irradiation-induced apoptosis. J. Virol. **77**:2063-2070.

67. **Arunagiri, C., I. Macreadie, D. Hewish, and A. A. Azad.** 1997. A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. Apoptosis **2**:69-76.

68. **Zhao, Y., M. Yu, M. Chen, R. T. Elder, A. Yamamoto, and J. Cao.** 1998. Pleiotropic effects of HIV-1 protein R (Vpr) on morphogenesis and cell survival in fission yeast and antagonism by pentoxifylline. Virology **246**:266-276.

69. **Nishizawa, M., M. Kamata, T. Mojin, Y. Nakai, and Y. Aida.** 2000. Induction of apoptosis by the Vpr protein of human immunodeficiency virus type 1 occurs independently of G(2) arrest of the cell cycle. Virology **276**:16-26.

70. **Watanabe, N., T. Yamaguchi, Y. Akimoto, J. B. Rattner, H. Hirano, and H. Nakauchi.** 2000. Induction of M-phase arrest and apoptosis after HIV-1 Vpr expression through uncoupling of nuclear and centrosomal cycle in HeLa cells. Exp. Cell Res. **258**:261-269.

71. **Chang, L. J., C. H. Chen, V. Urlacher, and T. Z. Lee.** 2000. Differential apoptosis effects of primate lentiviral Vpr and Vpx in mammalian cells. J. Biomed. Sci. **7**:322-333.

72. **Andersen, J. L., E. S. Zimmerman, J. L. DeHart, S. Murala, O. Ardon, J. Blackett, J. Chen, and V. Planelles.** 2005. ATR and GADD45alpha mediate HIV-1 Vpr-induced apoptosis. Cell Death Differ.**12**:326-334.

73. **Glynn, J. M., D. L. McElligott, and D. E. Mosier.** 1996. Apoptosis induced by HIV infection in H9 T cells is blocked by ICE-family protease inhibition but not

by a Fas(CD95) antagonist. J. Immunol. **157**:2754-2758.

74. **Abraham, R. T.** 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. **15**:2177-2196.

75. **Nyberg, K. A., R. J. Michelson, C. W. Putnam, and T. A. Weinert.** 2002. Toward maintaining the genome: DNA damage and replication checkpoints. Annu. Rev. Genet. **36**:617-656.

76. **Zhou, B. B. and S. J. Elledge.** 2000. The DNA damage response: putting checkpoints in perspective. Nature **408**:433-439.

77. **Siede, W., A. S. Friedberg, I. Dianova, and E. C. Friedberg.** 1994. Characterization of G1 checkpoint control in the yeast Saccharomyces cerevisiae following exposure to DNA-damaging agents. Genetics **138**:271-281.

78. **Garvik, B., M. Carson, and L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell Biol. **15**:6128-6138.

79. **Lopez-Aviles, S., M. Grande, M. Gonzalez, A. L. Helgesen, V. Alemany, M. Sanchez-Piris, O. Bachs, J. B. Millar, and R. Alique.** 2005. Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast. Mol. Cell **17**:49-59.

80. **Manke, I. A., A. Nguyen, D. Lim, M. Q. Stewart, A. E. Elia, and M. B. Yaffe.** 2005. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. Mol. Cell **17**:37-48.

81. **Tzivion, G., Y. H. Shen, and J. Zhu.** 2001. 14-3-3 proteins; bringing new

definitions to scaffolding. Oncogene **20**:6331-6338.

82. **Bohm, L., W. P. Roos, and A. M. Serafin.** 2003. Inhibition of DNA repair by Pentoxifylline and related methylxanthine derivatives. Toxicology **193**:153-160.

83. **Zou, L., D. Cortez, and S. J. Elledge.** 2002. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev. **16**:198-208.

84. **Zhou, Y. and L. Ratner.** 2000. Phosphorylation of human immunodeficiency virus type 1 Vpr regulates cell cycle arrest. J. Virol. **74**:6520-6527.

85. **Ward, I. M. and J. Chen.** 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J. Biol. Chem. **276**:47759-47762.

86. **Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner.** 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. **273**:5858-5868.

87. **Burma, S., B. P. Chen, M. Murphy, A. Kurimasa, and D. J. Chen.** 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J. Biol. Chem. **276**:42462-42467.

88. **Pilch, D. R., O. A. Sedelnikova, C. Redon, A. Celeste, A. Nussenzweig, W. M. Bonner.** 2003. Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem. Cell Biol. **81**:123-129.

89. **Furuta, T., H. Takemura, Z. Y. Liao, G. J. Aune, C. Redon, O. A. Sedelnikova, D. R. Pilch, E. P. Rogakou, A. Celeste, H. D. Chen, A. Nussenzweig, M. I. Aladjem, W. M. Bonner, and Y. Pommier.** 2003.

Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. J. Biol. Chem. **278**:20303-20312.

90. **Bartz, S. R., M. E. Rogel, and M. Emerman.** 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. J. Virol. **70**:2324-2331.

91. **Masuda, M., Y. Nagai, N. Oshima, K. Tanaka, H. Murakami, H. Igarashi, and H. Okayama.** 2000. Genetic studies with the fission yeast Schizosaccharomyces pombe suggest involvement of wee1, ppa2, and rad24 in induction of cell cycle arrest by human immunodeficiency virus type 1 Vpr. J. Virol. **74**:2636-2646.

92. **Elder, R. T., M. Yu, M. Chen, S. Edelson, and Y. Zhao.** 2000. Cell cycle G2 arrest induced by HIV-1 Vpr in fission yeast (Schizosaccharomyces pombe) is independent of cell death and early genes in the DNA damage checkpoint. Virus Res. **68**:161-173.

93. **Elder, R. T., M. Yu, M. Chen, X. Zhu, M. Yanagida, and Y. Zhao.** 2001. HIV-1 Vpr induces cell cycle G2 arrest in fission yeast (Schizosaccharomyces pombe) through a pathway involving regulatory and catalytic subunits of PP2A and acting on both Wee1 and Cdc25. Virology **287**:359-370.

94. **Poon, B., J. B. Jowett, S. A. Stewart, R. W. Armstrong, G. M. Rishton, and I. S. Chen.** 1997. Human immunodeficiency virus type 1 vpr gene induces

phenotypic effects similar to those of the DNA alkylating agent, nitrogen mustard. J. Virol. **71**:3961-3971.

95. **Zhu, Y., H. A. Gelbard, M. Roshal, S. Pursell, B. D. Jamieson, and V. Planelles.** 2001. Comparison of cell cycle arrest, transactivation, and apoptosis induced by the simian immunodeficiency virus SIVagm and human immunodeficiency virus type 1 vpr genes. J. Virol. **75**:3791-3801.

96. **Roshal, M., B. Kim, Y. Zhu, P. Nghiem, and V. Planelles.** 2003. Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R. J. Biol. Chem. **278**:25879-25886.

97. **Zimmerman, E. S., J. Chen, J. L. Andersen, O. Ardon, J. L. Dehart, J. Blackett, S. K. Choudhary, D. Camerini, P. Nghiem, and V. Planelles.** 2004. Human immunodeficiency virus type 1 Vpr-mediated G2 arrest requires Rad17 and Hus1 and induces nuclear BRCA1 and gamma-H2AX focus formation. Mol. Cell Biol. **24**:9286-9294.

98. **de Noronha, C. M., M. P. Sherman, H. W. Lin, M. V. Cavrois, R. D. Moir, R. D. Goldman, and W. C. Greene.** 2001. Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. Science **294**:1105-1108.

99. **Goh, W. C., N. Manel, and M. Emerman.** 2004. The human immunodeficiency virus Vpr protein binds Cdc25C: implications for G2 arrest. Virology **318**:337-349.

100. **Zhao, R. Y. and R. T. Elder.** 2005. Viral infections and cell cycle G2/M regulation. Cell Res. **15**:143-149.

101. **Nunnari, G., E. Argyris, J. Fang, K. E. Mehlman, R. J. Pomerantz, and R.**

Daniel. 2005. Inhibition of HIV-1 replication by caffeine and caffeine-related methylxanthines. Virology **335**:177-184.

102. **Zhao, Y., J. Cao, M. R. O'Gorman, M. Yu, and R. Yogev.** 1996. Effect of human immunodeficiency virus type 1 protein R (vpr) gene expression on basic cellular function of fission yeast Schizosaccharomyces pombe. J. Virol. **70**:5821-5826.

103. **Zhang, C., C. Rasmussen, and L. J. Chang.** 1997. Cell cycle inhibitory effects of HIV and SIV Vpr and Vpx in the yeast Schizosaccharomyces pombe. Virology **230**:103-112.

104. **Yuan, H., M. Kamata, Y. M. Xie, and I. S. Chen.** 2004. Increased levels of Wee-1 kinase in G(2) are necessary for Vpr- and gamma irradiation-induced G(2) arrest. J. Virol. **78**:8183-8190.

105. **Kino, T., A. Gragerov, A. Valentin, M. Tsopanomihalou, G. Ilyina-Gragerova, R. Erwin-Cohen, G. P. Chrousos, and G. N. Pavlakis.** 2005. Vpr protein of human immunodeficiency virus type 1 binds to 14-3-3 proteins and facilitates complex formation with Cdc25C: implications for cell cycle arrest. J. Virol. **79**:2780-2787.

106. **Kino, T., M. U. De Martino, E. Charmandari, T. Ichijo, T. Outas, and G. P. Chrousos.** 2005. HIV-1 accessory protein Vpr inhibits the effect of insulin on the Foxo subfamily of forkhead transcription factors by interfering with their binding to 14-3-3 proteins: potential clinical implications regarding the insulin resistance of HIV-1-infected patients. Diabetes **54**:23-31.

107. **Pozuelo Rubio, M., K. M. Geraghty, B. H. Wong, N. T. Wood, D. G.**

Campbell, N. Morrice, and C. Mackintosh. 2004. 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. Biochem. J. **379**:395-408.

108. **Dunand-Sauthier, I., C. Walker, C. Wilkinson, C. Gordon, R. Crane, C. Norbury, and T. Humphrey.** 2002. Sum1, a component of the fission yeast eIF3 translation initiation complex, is rapidly relocalized during environmental stress and interacts with components of the 26S proteasome. Mol. Biol. Cell **13**:1626-1640.

109. **Humphrey, T. and T. Enoch.** 1998. Sum1, a highly conserved WD-repeat protein, suppresses S-M checkpoint mutants and inhibits the osmotic stress cell cycle response in fission yeast. Genetics **148**:1731-1742.

110. **Wong, H. R., K. E. Dunsmore, K. Page, and T. P. Shanley.** 2005. Heat shock-mediated regulation of MKP-1. Am. J. Physiol. Cell Physiol. **289**:C1152-1158.

111. **Pratt, W. B., M. D. Galigniana, J. M. Harrell, and D. B. DeFranco.** 2004. Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. Cell Signal. **16**:857-872.

112. **Pratt, W. B., M. D. Galigniana, Y. Morishima, and P. J. Murphy.** 2004. Role of molecular chaperones in steroid receptor action. Essays Biochem. **40**:41-58.

113. **Pratt, W. B. and D. O. Toft**. 2003. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp. Biol. Med. (Maywood) **228**:111-133.

114. **Pratt, W. B., A. M. Silverstein, and M. D. Galigniana.** 1999. A model for the cytoplasmic trafficking of signalling proteins involving the hsp90-binding

immunophilins and p50cdc37. Cell Signal. **11**:839-851.

115. **Kotlyarov, A. and M. Gaestel.** 2002. Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression? Biochem. Soc. Trans. **30**:959-963.

116. **Stokoe, D., K. Engel, D. G. Campbell, P. Cohen, and M. Gaestel.** 1992. Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Lett. **313**:307-313.

117. **Landry, J. and J. Huot.** 1995. Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. Biochem. Cell Biol. **73**:703-707.

118. **Cairns, J., S. Qin, R. Philp, Y. H. Tan, and G. R. Guy.** 1994. Dephosphorylation of the small heat shock protein Hsp27 in vivo by protein phosphatase 2A. J. Biol. Chem. **269**:9176-9183.

119. **Wang, X., N. Grammatikakis, A. Siganou, M. A. Stevenson, and S. K. Calderwood.** 2004. Interactions between extracellular signal-regulated protein kinase 1, 14-3-3epsilon, and heat shock factor 1 during stress. J. Biol. Chem. **279**:49460-49469.

120. **Gu, J., M. Emerman, and S. Sandmeyer.** 1997. Small heat shock protein suppression of Vpr-induced cytoskeletal defects in budding yeast. Mol. Cell Biol. **17**:4033-4042.

121. **Macreadie, I. G., L. A. Castelli, D. R. Hewish, A. Kirkpatrick, A. C. Ward, and A. A. Azad.** 1995. A domain of human immunodeficiency virus type 1 Vpr

containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc. Natl. Acad. Sci. USA **92**:2770-2774.

122. **Benko, Z., D. Liang, E. Agbottah, J. Hou, K. Chiu, M. Yu, S. Innis, P. Reed, W. Kabat, R. T. Elder, P. Di Marzio, L. Taricani, L. Ratner, P. G. Young, M. Bukrinsky, and R. Y. Zhao.** 2004. Anti-Vpr activity of a yeast chaperone protein. J. Virol. **78**:11016-11029.

123. **Bukrinsky, M. and Y. Zhao.** 2004. Heat-shock proteins reverse the G2 arrest caused by HIV-1 viral protein R. DNA Cell Biol. **23**:223-225.

124. **Munoz, M. J., E. R. Bejarano, R. R. Daga, and J. Jimenez.** 1999. The identification of Wos2, a p23 homologue that interacts with Wee1 and Cdc2 in the mitotic control of fission yeasts. Genetics **153**:1561-1572.

125. **Munoz, M. J. and J. Jimenez.** 1999. Genetic interactions between Hsp90 and the Cdc2 mitotic machinery in the fission yeast Schizosaccharomyces pombe. Mol. Gen. Genet. **261**:242-250.

126. **Johnson, J. L., T. G. Beito, C. J. Krco, and D. O. Toft.** 1994. Characterization of a novel 23-kilodalton protein of unactive progesterone receptor complexes. Mol. Cell Biol. **14**:1956-1963.

127. **Goes, F. S. and J. Martin.** 2001. Hsp90 chaperone complexes are required for the activity and stability of yeast protein kinases Mik1, Wee1 and Swe1. Eur. J. Biochem. **268**:2281-2289.

128. **Tanioka, T., Y. Nakatani, N. Semmyo, M. Murakami, and I. Kudo.** 2000. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2

biosynthesis. J. Biol. Chem. **275**:32775-32782.

129. **Basu, S., R. J. Binder, R. Suto, K. M. Anderson, and P. K. Srivastava.** 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int. Immunol. **12**:1539-1546.

130. **Asea, A., S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, R. W. Finberg, G. C. Koo, and S. K. Calderwood.** 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med. **6**:435-442.

131. **Asea, A., M. Rehli, E. Kabingu, J. A. Boch, O. Bare, P. E. Auron, M. A. Stevenson, and S. K. Calderwood.** 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. **277**:15028-15034.

132. **Lipsker, D., U. Ziylan, D. Spehner, F. Proamer, H. Bausinger, P. Jeannin, J. Salamero, A. Bohbot, J. P. Cazenave, R. Drillien, Y. Delneste, D. Hanau, and H. de la Salle.** 2002. Heat shock proteins 70 and 60 share common receptors which are expressed on human monocyte-derived but not epidermal dendritic cells. Eur. J. Immunol. **32**:322-332.

133. **Romisch, K.** 2001. Anti-bacterial defense relies on chaperone gp96. Trends Biochem. Sci. **26**:644.

134. **Vabulas, R.M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and H. Wagner.** 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem. **277**:15107-15112.

135. **Gurer, C., A. Cimarelli, and J. Luban.** 2002. Specific incorporation of heat shock protein 70 family members into primate lentiviral virions. J. Virol. **76**:4666-4670.

136. **Furlini, G., M. Vignoli, M. C. Re, D. Gibellini, E. Ramazzotti, G. Zauli, and M. La Placa.** 1994. Human immunodeficiency virus type 1 interaction with the membrane of CD4+ cells induces the synthesis and nuclear translocation of 70K heat shock protein. J. Gen. Virol. **75**:193-199.

137. **Wainberg, Z., M. Oliveira, S. Lerner, Y. Tao, and B. G. Brenner.** 1997. Modulation of stress protein (hsp27 and hsp70) expression in CD4+ lymphocytic cells following acute infection with human immunodeficiency virus type-1. Virology **233**:364-373.

138. **Agostini, I., S. Popov, J. Li, L. Dubrovsky, T. Hao, and M. Bukrinsky.** 2000. Heat-shock protein 70 can replace viral protein R of HIV-1 during nuclear import of the viral preintegration complex. Exp. Cell Res. **259**:398-403.

139. **Iordanskiy, S., Y. Zhao, P. DiMarzio, I. Agostini, L. Dubrovsky, and M. Bukrinsky.** 2004. Heat-shock protein 70 exerts opposing effects on Vpr-dependent and Vpr-independent HIV-1 replication in macrophages. Blood **104**:1867-1872.

140. **Iordanskiy, S., Y. Zhao, L. Dubrovsky, T. Iordanskaya, M. Chen, D. Liang, and M. Bukrinsky.** 2004. Heat shock protein 70 protects cells from cell cycle arrest and apoptosis induced by human immunodeficiency virus type 1 viral protein R. J. Virol. **78**:9697-9704.

141. **Sherman, M. P., C. M. de Noronha, S. A. Williams, and W. C. Greene.** 2002.

Insights into the biology of HIV-1 viral protein R. DNA Cell Biol. **21**:679-688.

142. **Chang, F., F. Re, S. Sebastian, S. Sazer, and J. Luban.** 2004. HIV-1 Vpr induces defects in mitosis, cytokinesis, nuclear structure, and centrosomes. Mol. Biol. Cell **15**:1793-1801.

143. **Jaspersen, S. L. and M. Winey.** 2004. The budding yeast spindle pole body: structure, duplication, and function. Annu. Rev. Cell Dev. Biol. **20**:1-28.

144. **Meraldi, P. and E. A. Nigg.** 2002. The centrosome cycle. FEBS Lett. **521**:9-13.

145. **Lange, B. M.** 2002. Integration of the centrosome in cell cycle control, stress response and signal transduction pathways. Curr. Opin. Cell Biol. **14**:35-43.

146. **Jackman, M., C. Lindon, E. A. Nigg, and J. Pines.** 2003. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat. Cell Biol. **5**:143-148.

147. **Xie, S., B. Xie, M. Y. Lee, and W. Dai.** 2005. Regulation of cell cycle checkpoints by polo-like kinases. Oncogene **24**:277-286.

148. **Glover, D. M.** 2005. Polo kinase and progression through M phase in Drosophila: a perspective from the spindle poles. Oncogene **24**:230-237.

149. **Glover, D. M., I. M. Hagan, and A. A. Tavares.** 1998. Polo-like kinases: a team that plays throughout mitosis. Genes Dev. **12**:3777-3787.

150. **Ohkura, H., I. M. Hagan, and D. M. Glover.** 1995. The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev. **9**:1059-1073.

151. **Chen, M. S., J. Hurov, L. S. White, T. Woodford-Thomas, and H. Piwnica-Worms.** 2001. Absence of apparent phenotype in mice lacking Cdc25C protein

phosphatase. Mol. Cell Biol. **21**:3853-3861.

152. **Nishizawa, M., T. Myojin, Y. Nishino, Y. Nakai, M. Kamata, and Y. Aida.** 1999. A carboxy-terminally truncated form of the Vpr protein of human immunodeficiency virus type 1 retards cell proliferation independently of G(2) arrest of the cell cycle. Virology **263**:313-322.

153. **Nishino, Y., T. Myojin, M. Kamata, and Y. Aida.** 1997. Human immunodeficiency virus type 1 Vpr gene product prevents cell proliferation on mouse NIH3T3 cells without the G2 arrest of the cell cycle. Biochem. Biophys. Res. Commun. **232**:550-554.

154. **Chan, T. A., P. M. Hwang, H. Hermeking, K. W. Kinzler, and B. Vogelstein.** 2000. Cooperative effects of genes controlling the G(2)/M checkpoint. Genes Dev. **14**:1584-1588.

155. **Hermeking, H., C. Lengauer, K. Polyak, T. C. He, L. Zhang, S. Thiagalingam, K. W. Kinzler, and B. Vogelstein.** 1997. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol. Cell **1**:3-11.

156. **Dellambra, E., M. Patrone, B. Sparatore, A. Negri, F. Ceciliani, S. Bondanza, F. Molina, F. D. Cancedda, and M. De Luca.** 1995. Stratifin, a keratinocyte specific 14-3-3 protein, harbors a pleckstrin homology (PH) domain and enhances protein kinase C activity. J. Cell Sci. **108**:3569-3579.

157. **Leffers, H., P. Madsen, H. H. Rasmussen, B. Honore, A. H. Andersen, E. Walbum, J. Vandekerckhove, and J. E. Celis.** 1993. Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signalling pathway.

J. Mol. Biol. **231**:982-998.

158. **Hrimech, M., X. J. Yao, P. E. Branton, and E. A. Cohen.** 2000. Human immunodeficiency virus type 1 Vpr-mediated G(2) cell cycle arrest: Vpr interferes with cell cycle signaling cascades by interacting with the B subunit of serine/threonine protein phosphatase 2A. EMBO J. **19**:3956-3967.

159. **Dougherty, M. K. and D. K. Morrison.** 2004. Unlocking the code of 14-3-3. J. Cell Sci. **117**:1875-1884.

160. **Huang, H. K., S. L. Forsburg, U. P. John, M. J. O'Connell, and T. Hunter.** 2001. Isolation and characterization of the Pin1/Ess1p homologue in Schizosaccharomyces pombe. J. Cell Sci. **114**:3779-3788.

161. **Karaiskou, A., C. Jessus, T. Brassac, and R. Ozon.** 1999. Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. J. Cell Sci. **112**:3747-3756.

162. **Rime, H., C. Jessus, and R. Ozon.** 1995. Tyrosine phosphorylation of p34cdc2 is regulated by protein phosphatase 2A in growing immature Xenopus oocytes. Exp. Cell Res. **219**:29-38.

163. **Che, S., W. Wu, M. Nelman-Gonzalez, T. Stukenberg, R. Clark, and J. Kuang.** 1998. A phosphatase activity in Xenopus oocyte extracts preferentially dephosphorylates the MPM-2 epitope. FEBS Lett. **424**:225-233.

164. **Lin, F. C. and K. T. Arndt.** 1995. The role of Saccharomyces cerevisiae type 2A phosphatase in the actin cytoskeleton and in entry into mitosis. EMBO J. **14**:2745-2759.

165. **Shimura, M., Y. Tanaka, S. Nakamura, Y. Minemoto, K. Yamashita, K.**

Hatake, F. Takaku, and Y. Ishizaka. 1999. Micronuclei formation and aneuploidy induced by Vpr, an accessory gene of human immunodeficiency virus type 1. FASEB J. **13**:621-637.

166. **Shimura, M., Y. Onozuka, T. Yamaguchi, K. Hatake, F. Takaku, and Y. Ishizaka.** 1999. Micronuclei formation with chromosome breaks and gene amplification caused by Vpr, an accessory gene of human immunodeficiency virus. Cancer Res. **59**:2259-2264.

167. **Minemoto, Y., M. Shimura, Y. Ishizaka, Y. Masamune, and K. Yamashita.** 1999. Multiple centrosome formation induced by the expression of Vpr gene of human immunodeficiency virus. Biochem. Biophys. Res. Commun. **258**:379-384.

168. **Mahalingam, S., V. Ayyavoo, M. Patel, T. Kieber-Emmons, G. D. Kao, R. J. Muschel, and D. B. Weiner.** 1998. HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G2/M phase transition of the mammalian cell cycle. Proc. Natl. Acad. Sci. USA **95**:3419-3424.

169. **Ramanathan, M. P., E. Curley III, M. Su, J. A. Chambers, and D. B. Weiner.** 2002. Carboxyl terminus of hVIP/mov34 is critical for HIV-1-Vpr interaction and glucocorticoid-mediated signaling. J. Biol. Chem. **277**:47854-47860.

170. **Schrofelbauer, B., Q. Yu, S. G. Zeitlin, and N. R. Landau.** 2005. Human immunodeficiency virus type 1 Vpr induces the degradation of the UNG and SMUG uracil-DNA glycosylases. J. Virol. **79**:10978-10987.

171. **Zhao, L. J., H. Jian, and H. Zhu.** 2004. HIV-1 auxiliary regulatory protein Vpr promotes ubiquitination and turnover of Vpr mutants containing the L64P mutation. FEBS Lett. **563**:170-178.

172. **Chen, R., E. Le Rouzic, J. A. Kearney, L. M. Mansky, and S. Benichou.** 2004. Vpr-mediated incorporation of UNG2 into HIV-1 particles is required to modulate the virus mutation rate and for replication in macrophages. J. Biol. Chem. **279**:28419-28425.

173. **Selig, L., S. Benichou, M. E. Rogel, L. I. Wu, M. A.Vodicka, J. Sire, R. Benarous, and M. Emerman.** 1997. Uracil DNA glycosylase specifically interacts with Vpr of both human immunodeficiency virus type 1 and simian immunodeficiency virus of sooty mangabeys, but binding does not correlate with cell cycle arrest. J. Virol. **71**:4842-4846.

174. **Dieckmann, T., E. S. Withers-Ward, M. A. Jarosinski, C. F. Liu, I. S. Chen, and J. Feigon.** 1998. Structure of a human DNA repair protein UBA domain that interacts with HIV-1 Vpr. Nat. Struct. Biol. **5**:1042-1047.

175. **Gaynor, E. M. and I. S. Chen.** 2001. Analysis of apoptosis induced by HIV-1 Vpr and examination of the possible role of the hHR23A protein. Exp. Cell Res. **267**:243-257.

176. **Gragerov, A., T. Kino, G. Ilyina-Gragerova, G. P. Chrousos, and G. N. Pavlakis.** 1998. HHR23A, the human homologue of the yeast repair protein RAD23, interacts specifically with Vpr protein and prevents cell cycle arrest but not the transcriptional effects of Vpr. Virology **245**:323-330.

177. **Withers-Ward, E. S., J. B. Jowett, S. A. Stewart, Y. M. Xie, A. Garfinkel, Y. Shibagaki, S. A. Chow, N. Shah, F. Hanaoka, D. G. Sawitz, R. W. Armstrong, L. M. Souza, and I. S. Chen.** 1997. Human immunodeficiency virus type 1 Vpr interacts with HHR23A, a cellular protein implicated in nucleotide

excision DNA repair. J. Virol. **71**:9732-9742.

178. **Withers-Ward, E. S., T. D. Mueller, I. S. Chen, and J. Feigon.** 2000. Biochemical and structural analysis of the interaction between the UBA(2) domain of the DNA repair protein HHR23A and HIV-1 Vpr. Biochemistry **39**:14103-14112.

179. **Matarrese, P., L. Conti, B. Varano, M. C. Gauzzi, F. Belardelli, S. Gessani, and W. Malorni.** 2000. The HIV-1 vpr protein induces anoikis-resistance by modulating cell adhesion process and microfilament system assembly. Cell Death Differ. **7**:25-36.

180. **Stantchev, T. S. and C. C. Broder.** 2001. Human immunodeficiency virus type-1 and chemokines: beyond competition for common cellular receptors. Cytokine Growth Factor Rev. **12**:219-243.

181. **Hrimech, M., X. J. Yao, F. Bachand, N. Rougeau, and E. A. Cohen.** 1999. Human immunodeficiency virus type 1 (HIV-1) Vpr functions as an immediate-early protein during HIV-1 infection. J. Virol. **73**:4101-4109.

182. **Kino, T., A. Gragerov, J. B. Kopp, R. H. Stauber, G. N. Pavlakis, and G. P. Chrousos.** 1999. The HIV-1 virion-associated protein vpr is a coactivator of the human glucocorticoid receptor. J. Exp. Med. **189**:51-62.

183. **Kino, T., J. B. Kopp, and G. P. Chrousos.** 2000. Glucocorticoids suppress human immunodeficiency virus type-1 long terminal repeat activity in a cell type-specific, glucocorticoid receptor-mediated fashion: direct protective effects at variance with clinical phenomenology. J. Steroid Biochem. Mol. Biol. **75**:283-290.

184. **Mirani, M., I. Elenkov, S. Volpi, N. Hiroi, G. P. Chrousos, and T. Kino.** 2002. HIV-1 protein Vpr suppresses IL-12 production from human monocytes by enhancing glucocorticoid action: potential implications of Vpr coactivator activity for the innate and cellular immunity deficits observed in HIV-1 infection. J. Immunol. **169**:6361-6368.

185. **Fakruddin, J. M. and J. Laurence.** 2005. HIV-1 Vpr enhances production of receptor of activated NF-kappaB ligand (RANKL) via potentiation of glucocorticoid receptor activity. Arch. Virol. **150**:67-78.

186. **Kinoshita, N., H. Yamano, H. Niwa, T. Yoshida, and M. Yanagida.** 1993. Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. Genes Dev. **7**:1059-1071.

187. **Rime, H., C. Jessus, and R. Ozon.** 1995. Tyrosine phosphorylation of p34cdc2 is regulated by protein phosphatase 2A in growing immature Xenopus oocytes. Exp. Cell Res. **219**:29-38.

188. **Karaiskou, A., C. Jessus, T. Brassac, and R. Ozon.** 1999. Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. J. Cell Sci. **112**:3747-3756.

189. **Yang, H., W. Jiang, M. Gentry, and R. L. Hallberg.** 2000. Loss of a protein phosphatase 2A regulatory subunit (Cdc55p) elicits improper regulation of Swe1p degradation. Mol. Cell Biol. **20**:8143-8156.

190. **Zhou, X. Z., O. Kops, A. Werner, P. J. Lu, M. Shen, G. Stoller, G. Kullertz, M. Stark, G. Fischer, and K. P. Lu.** 2000. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Mol. Cell **6**:873-883.

191. **Tang Z., T. R. Coleman, and W. G. Dunphy.** 1993. Two distinct mechanisms for negative regulation of the Wee1 protein kinase. EMBO J. **12**:3427-3436.

192. **Michael, W. M. and J. Newport.** 1998. Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. Science **282**:1886-1889.